INFLUENCE OF OXYGEN TENSION AND CONCENTRATION ON BONE AND CARTILAGE RUDIMENTS IN TISSUE CULTURE

by

David Lawrence Hamblen, M.B., B.S. (Lond.)

Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine.

July, 1974.

INFLUENCE OF OXYGEN TENSION AND CONCENTRATION ON BONE AND CARTILAGE RUDIMENTS IN TISSUE CULTURE

David Lawrence Hamblen

ERRATA LIST

Page	Line	
27	26	50 µgm/ml ascorbic acid
29	1	50 µgm/ml
	12	5 µgm/ml
	15	150 µgm/ml
106	23	560 mµ
107	18	2 and 15 µgms/ml
110	9	1.0 µCi/ml
138	26	7.5 µg/ml
139	19	0.1 µg/ml
	23	1.0 µg/ml
140	1	10 - 100 µg/ml
	6	10 µg/ml
	14	1.0 µg/ml
	17	0.01 µg/ml
144	5	1.0 or 5.0 µgm/ml

INDEX

SUMMARY OF WORK

A stationary organ culture technique has been used to study the effects of increased oxygen concentration and pressure on late foetal mouse limb bone rudiments.

Preliminary experiments were performed to determine the culture conditions necessary for optimum growth of rudiments in air over a six day period. The influence of the choice of culture medium, addition of serum supplements, and level of ascorbic acid on explant survival and growth was fully evaluated. A comparison was made between the growth and development of the embryonic tibia, radius and ulna, in vivo and in vitro. Particular attention was paid to histological changes with the use of histochemical techniques to identify alterations in the matrix of cartilage and bone as well as the cells themselves.

The same histological techniques were then used to compare the in vitro growth in air with that in oxygen at atmospheric and hyperbaric pressures. Exposure to oxygen at three atmospheres absolute pressure resulted in death of the rudiments, but some viability could be maintained at two atmospheres. Rudiments grown in hyperbaric oxygen showed some of the increased degradation of cartilage and bone normally obtained with hyperoxygenation at atmospheric pressure. In addition, there was an increased thickening of the periosteal osteoblast layer of the shaft in the early stages, but this was not maintained.

Using tritium labelled proline incorporated in the culture media a biochemical and radiochemical analysis was made

of the synthesis and degradation of hydroxyproline, and thus collagen, in the rudiments during six days in culture. The synthesis of new collagen was highest in air, less active in hyperbaric oxygen and lowest in oxygen at normal pressure, while degradation occurred in the opposite sequence.

Rudiments grown on media containing tritiated proline were examined by autoradiography to determine the site of new collagen formation and resorption. Labelling was heaviest in relation to the deep cells of the periosteum, particularly in air, but also occurred in the end cartilages where it was heaviest in the proliferative cell layer. Some grains were deposited in the osteoid formed on the surface of the cartilage cores in the metaphyseal region, suggesting that endochondral bone formation can continue in vitro. Resorption of labelled bone matrix was less in hyperbaric oxygen than in conditions of hyperoxia at normobaric pressure.

Lysosomal enzymes have been suggested as the cuase of bone and cartilage resorption under conditions of hyperoxia. Free and bound enzyme was assayed biochemically in the culture medium and the rudiments themselves. The free activity of acid protease was increased in both rudiments and media grown in oxygen, but fell to a level nearer that of the controls in air under hyperbaric oxygenation.

Experiments adding hydrocortisone or E-amino caproic acid to the culture medium were used to test their ability to stabilise lysosomal membranes or block the action of released enzymes. Both substances limited the degradation of cartilage induced by

hyperoxia but could not prevent the resorption of bone. The free activity of lysosomal enzymes was reduced by the presence of hydrocortisone in the medium but this did not parallel the resorption of bone.

It is concluded the lysosomal enzymes may have a role in the degradation of cartilage induced by hyperoxia, but that other factors must play an additional part in the resorption of bone. In 95% oxygen at atmospheric pressure the resorption of bone and cartilage in the metaphyseal region was accompanied by the appearance of numerous osteoclasts in the first day, but these later disappeared. The later stages of bone resorption in hyperoxia was characterised by marked osteocytic osteolysis, which is less directly linked with lysosomal enzyme release. The toxic effects of hyperbaric oxygen seemed to limit the extent of bone resorption by both these mechanism.

GENERAL INTRODUCTION

This thesis describes an investigation of the changes induced by alterations in oxygen concentration and pressure in late foetal mouse limb bone rudiments maintained in vitro.

Oxygen is necessary for the aerobic metabolism of tissues, but it is also thought to be an important environmental factor in the cellular differentiation of connective tissues, especially those of the skeleton. The evidence for this has been well reviewed by Hall (1970) who concluded that hyperoxia enhances collagen formation which in turn favours osteogenesis, while hypoxia enhances chondroitin sulphate formation which favours chondrogenesis.

Effects of oxygen on skeletal tissues in vivo.

In vivo studies have been based on experiments using oxygen at increased or decreased ambient pressures, since the full saturation of haemoglobin by oxygen at atmospheric pressure makes the state of oxygenation at tissue level entirely dependent on the local circulation. Severson and Tonna (1968) studied the periosteal proliferation following trauma to the femora of young mice, which were exposed to low oxygen tensions for 30 hours. Using an autoradiographic technique after in vivo tritium labelling, they reported no significant effect in 15% oxygen and only slight reduction in labelling with 10% oxygen at ambient pressure. They felt that the deficient oxygenation at the site of trauma was insufficient to stimulate a local tissue factor capable of inducing cell proliferation. Manspeizer and Tonna

(1967) had previously demonstrated that lowering the oxygen concentration to 5% eliminates cell proliferation because of oxygen debt.

Some indirect evidence can be obtained from the results of ischaemia or venous pooling produced in the skeleton experimentally or by disease. Brookes and Helal (1968) studied the pH of interosseous blood samples taken from the metaphysis and midshaft of the femur and tibia in young rabbits. They showed that the repair of experimental fractures, by abundant young osteoblastic tissue, was accelerated in hind limbs when venous congestion was produced. They also reported that the production of cartilage, normally seen at the fracture site in controls, was almost abolished. They noted that more active metaphyseal bone formation was associated with the more acid pH in the microcirculation. They felt that their results indicated that the formation of bone is increased in the presence of venous congestion and may be linked with reduced pH and oxygen tension or an increase in carbon dioxide tension consequent upon an impeded micro-circulation. It was suggested that the role of oxygen tension in an osteogenic region, considered together with the appropriate rate of blood flow, is probably related to the total amount of bone turnover, because of the energy-dependent nature of the protein synthesis involved in both bone formation and removal. Brighton and Heppenstall (1971) using a microelectrode recorded the oxygen tension in the zones of the epiphyseal plate, the metaphysis and diaphysis of young rabbits. In the epiphysis, the oxygen tension increases from 20.5 mm Hg

in the zone of small cartilage cells to 57.0 mm Hg in the zone of cell columns, falling again to 19.8 mm Hg in the hypertrophic cell zone and the adjacent metaphysis. In the diaphysis the oxygen tension again rose to 108.7 mm Hg. Their findings of a lower oxygen tension in the hypertrophic cell zone and metaphysis would fit the suggestion that chronic stasis and hypoxia are necessary for cartilage calcification and endochondral bone formation.

Hyperbaric oxygen and the skeleton.

Hyperbaric oxygen therapy, or the inhalation of oxygen at increased pressure, results in an increase in the quantity of oxygen carried in physical solution in the blood. Breathing air at atmospheric pressure gives an arterial oxygen tension of 100 mm Hg and the quantity of oxygen in physical solution in the blood is approximately 0.3 volumes per cent. This rises from 2.0 volumes per cent when breathing pure oxygen at atmospheric pressure to 6.2 volumes per cent in oxygen at three atmospheres absolute pressure. As the normal arterio-venous difference in the blood oxygen content is 6 volumes per cent, this quantity carried in physical solution is sufficient to maintain the oxygenation of most body tissues without the need for red cells or haemoglobin.

Clinically, hyperbaric oxygen has been used for the treatment of chronic pyogenic bone infection or osteomyelitis with some success (Slack et al, 1965, and Perrins et al, 1966). The therapy was based on the inhibition of aerobic bacterial growth observed in vitro when cultured in oxygen at increased pressure. Because of the short intermittent exposures in patients permitted by the toxic effects of the therapy it is unlikely that a significant

inhibitory effect could be produced on these bacteria in vivo. In a study of experimental staphylococcal bone infections of the rat tibia, the present author (Hamblen, 1968) demonstrated an improved healing rate with intermittent exposures to oxygen at two and three atmospheres absolute pressure. It was suggested that the beneficial effect was due to some alteration in the host's response to the disease and that this might result in an increase in the rate of resorption or revascularisation of sclerotic bone and dense scar tissue with an associated stimulus to normal repair by osteogenesis.

The effect of hyperbaric oxygenation on the healing of experimental fractures has been investigated in several animals with equivocal results. Coulson et al (1966) demonstrated an increased uptake of Calcium 45 and an improvement in tensile strength in the healing femur of rats receiving intermittent therapy at three atmospheres pressure. This improved healing rate was confirmed by Yablon & Cruess (1968), who reported a greater production of new bone, both in the medullary region and subperiosteally, by conversion of the increased cartilage formed in the fracture callus. However, when the duration of hyperbaric treatment was increased to six hours daily at two atmospheres absolute, Wray and Rogers (1968) reported a decreased breaking strength in the fracture callus, though this was twice as active histologically as the normal control. The biochemical changes in the fracture callus from rats receiving hyperbaric oxygenation was investigated by Niinikoski et al (1970). They reported an increase in calcium, phosphorous, hexosamine, hydroxyproline and

nitrogen content, when compared with controls after eleven days of treatment, and suggested that the synthesis of proteins seemed to be increased. Persson (1967) studied the effect of hyperbaric oxygenation on the epiphyseal growth plate and growth in length of the rabbit tibia using an intra-vital tetracycline label. He found an acceleration of growth by 2% during the day of treatment followed by a deceleration of 7% on the subsequent day.

Oxygen effects in vitro.

Because of the complex interrelationships of oxygen and carbon dioxide tension and blood pH in the intact animal a more direct investigation by in vitro techniques would seem desirable. Following the first successful tissue culture by Ross Harrison (1907) techniques were quickly developed which allowed the maintenance of cells, tissues and organs in vitro with continuation of normal function. The term organ culture was introduced by Maximow (1925) to describe those experiments in explantation that tended to conserve in vitro the organic integrity of the rudiment. All methods of growing skeletal rudiments in vitro are based on the original classical method of Fell & Robison (1929). They had originally used a solid medium, such as a plasma clot, but this was later modified to allow use of either 'natural' or 'synthetic' culture media. Both bone and cartilage can be maintained in a healthy state at a gas-liquid interphase supported by a floating lens paper or a stainless steel grid. With the development of closed culture chambers by Trowell (1958) it became possible to control accurately the gaseous phase of the

tissue culture model. While allowing more accurate study of the effects of altered oxygen concentration it also permitted the maintenance of differentiated tissues in culture, where previously only embryonic organs could survive. Using variations of these methods many authors have studied the effects of altered oxygen tension on the metabolism of cartilage and bone.

Goldhaber (1958) studied foetal mouse calvaria and was able to demonstrate considerable resorption of the bone by exposure to 95% oxygen which did not occur when the cultures were maintained in air. The resorption was associated with an initial increase in macrophage cells and later osteoclast activity, while newly-formed osteoid tissue was also demonstrated in the cultures after resorption had ceased. In later experiments using a roller-tube apparatus in place of stationary cultures, Goldhaber (1963) reported almost complete resorption of the bone in 20% oxygen.

Using a different model of the chick-embryo cartilaginous limb bone rudiment, Sledge & Dingle (1965) demonstrated a reduction in longitudinal growth, a loss of metachromatic material, an increased release of protease and acid phosphatase enzymes, and a thickening in the periosteal osteoblast layer on exposure to 85% oxygen. They attributed these changes to activation of lysosomes by the hyperoxia and suggested that this was a prerequisite to the invasion of cartilage by vascular tissue which occurs in normal bone formation. Using a similar model Shaw & Bassett (1967) showed that maximum osteogenesis and collagen fibre formation occurred with a concentration of 35% oxygen. These processes were suppressed by lowering the oxygen

concentration to 5%, or to a less extent by raising it to 95%. Similar changes in cartilage matrix formation and resorption were associated with variations in oxygen tension. By these studies they were able to demonstrate that both bone and cartilage cells are exquisitely sensitive to the amount of available oxygen and that their behaviour ranges from synthetic to lytic activity.

Studies of the effect of hyperbaric oxygenation on tissue cultures have been few and confined to non-osseous tissue. Heppleston & Simnett (1964) exposed young mouse tissues in culture to 95% oxygen and 5% carbon dioxide at two atmospheres absolute pressure. They showed that damage occurred to the epithelium of pulmonary alveoli and renal tubules more readily than those to bronchi, epididymis or prostate. However, Stier & Halasz (1966) attributed these toxic effects to the acidosis produced in the culture medium from the use of too high a concentration of carbon dioxide and reported long-term tissue survival using a gaseous phase of 98% oxygen with 2% carbon dioxide. They demonstrated survival in adult tissues over a radius of 1.5 cm for 10-14 days using increased oxygen pressures up to four atmospheres absolute.

In the work to be described these studies have been extended to the effects of increased oxygen concentration and pressure on mammalian embryonic limb bone rudiments. Late foetal mouse long bones were chosen because the previous work studying the effects of hyperoxia has been confined to the avian limb bone and species differences may exist. There is also a suggestion (Mitchell, 1950)

that the degree of self differentiation in avian limb bones is much higher than in mammals making the interpretation of effects produced by other factors more difficult. In addition these rudiments offer a combined cartilage and bone model allowing the effects to be studied in both tissues. The general morphology of the bones in the fore and hind limbs of late embryonic mice is shown in Figure 1. The joints are developed in a rudimentary form allowing disarticulation by dissection in the preparation of individual bones for explantation. The bones themselves consist of a central bony shaft with a developing marrow cavity closed at either end by a cartilaginous epiphysis which stains densely with metachromatic stains such as toluidine blue (Fig.2).

Morphology of foetal and early postnatal mouse limb bone rudiments.

Accurate ageing of the mouse embryos used for explantation may be difficult when pregnant females are obtained from a commercial source. Even with the technique of breeding described in the Methods and Materials Section of Part 1 of this thesis, variations of up to half a day may occur. Another variable factor is introduced by the size of litter, as when the number of embryos was large skeletal development could be retarded by up to one day. For these reasons the rudiments used throughout this study were identified by a common nomenclature. This classifies the foetal rudiments as 'early', 'intermediate' or 'late', while in some experiments explants from one or two day old newborn mice were used and described as postnatal. The criteria for this classification and a description of the characteristic structure for each

type of rudiment will now be given.

(1) Early foetal rudiments.

Limb bone rudiments of tibia, radius and ulna were classified under this heading when they were largely cartilaginous in nature. An arbitrary maximum length was selected for bones at the time of explantation of 2.8 mm for tibiae, 2.5 mm for radii, and 3.5 mm for ulnae. The typical histological appearance of such an explant is shown in Figure 3. No marrow cavity has developed in the shaft and the end cartilages are continuous with each other. The cartilage shows early organization into zones. The small rounded chondroblasts of the epiphysis pass into a layer of more flattened cells which will form the future proliferative zone. This zone is continuous with a layer of larger hypertrophic cells which meet at the centre of the diaphysis. The chondroblasts of the hypertrophic zone are swollen and are surrounded by less ground substance than in the proximal zones. A thin collar of periosteal bone is usually present at the centre of the shaft, (Fig.4) formed by the osteoblastic cells in the deep layers of the covering periosteum or perichondrium. At higher power (Fig.5) these cells can be seen commencing invasion of the hypertrophic zone prior to the formation of the marrow cavity and the establishment of endochondral ossification. The periosteum is continuous with the perichondrium surrounding the cartilaginous ends. This

early foetal limb bone rudiment corresponds to the stage of development in the 14 to 15 day embryo.

(2) Intermediate foetal rudiment.

This stage of development corresponds to that normally seen in 16 to 17 day embryos. The overall length ranges are taken as 2.8 mm to 4.1 mm for the tibia, 2.6 mm to 3.4 mm for the radius, and 3.5 mm to 4.7 mm for the ulna. The end cartilages are now separated by a well developed marrow cavity enclosed between a bony shaft (Fig.6). The bone of the shaft occupies less than half the total length of the rudiment and is thickest at its centre. It shows a trabecular structure, with an endosteal layer on its deep surface separating it from the marrow cavity, which contains a mixed round cell population. The periosteum now shows two definite layers, an outer layer of flattened fibroblast-like cells and an inner layer of plumper osteoblastic cells laying down osteoid on their deep surface (Fig.7). The bone contains buried osteocyte cells lying in small lacunae and sometimes multinucleate osteoclasts are visible on the endosteal surface. The end cartilages show more differentiation into their individual zones (Fig.8), although there is still no sharp demarcation between the three types of cells. At both ends the rounded chondroblasts of the epiphysis become more flattened and arrange themselves in distinct columns in the proliferative zone where the ground substance is most abundant and deeply

stained by metachromatic techniques. The chondroblasts enlarge in the hypertrophic zone becoming more degenerate distally as they approach the marrow cavity.

(3) Late foetal rudiment.

The measurements of the late foetal rudiments fell in the range for the tibia 4.1 mm to 4.9 mm, for radius 3.4 mm to 4.0 mm and for the ulna 4.7 mm to 5.4 mm. The bony shaft is now well established and occupies more than half the total length of the rudiment as a tube of trabecular bone (Fig.9). The enlarged conical end cartilages show clear demarcation of their chondroblasts into the zones of epiphysis, proliferation, and hypertrophy, though the ground substance still stains most densely in the proliferative zone (Fig.10). The degenerate cartilage cells in the distal part of the hypertrophic zone are being eroded by capillaries from the ends of the bony shaft and the process of endochondral bone formation is well established. Islands of degenerate cartilage survive in the marrow of the adjacent metaphyseal region and are enclosed in the newly formed osteoid tissue of the trabecular bone (Fig.11). Periosteal bone formation is also well marked with active osteoid formation by the osteoblast cells in its deepest layers. This is most marked over the junction of bone and cartilage (Fig.12), while the thicker bone at the centre of the shaft is more dense and less trabecular.

(4) Postnatal limb bone rudiments.

These were similar in appearance to the late foetal rudiments, though the end cartilages were further narrowed by invasion of the marrow cavity with consequent increase in the length of the bony shaft. Endochondral bone formation continued actively but no secondary centre of ossification had appeared in the cartilage up to six days after birth. A fuller description of these rudiments will be given in Part II of this thesis comparing the growth of rudiments in vitro and in vivo.

Staining properties of limb bone rudiments.

The staining techniques used throughout this study for histological sections from the limb bone rudiments were chosen to demonstrate both structural and histochemical changes. Before using these techniques to evaluate changes occurring in the rudiments during culture, a review of the theoretical basis for their action on skeletal tissues was undertaken.

Haematoxylin and eosin staining (Lillie, 1965) was used as the standard general purpose method to give rapid identification of mesenchymal tissue structure (Fig.13). Mayer's haematoxylin was used in preference to Ehrlich's as it gives little or no staining of the mucopolysaccharide material in the cartilage.

Heidenhain's Azan method, a variant of the Masson trichrome stain, (Lillie, 1965) was used in addition to demonstrate the presence of young collagen fibrils, which stain an intense blue in contrast to the red coloration of cell nuclei, red blood cells,

and muscle (Fig.14).

Histochemical techniques were used for the differentiation of mucopolysaccharides in the cartilage and embryonic bone. The characterization of mammalian mucopolysaccharides has been well summarized by Spicer (1963) who recommended the use of a combined Alcian blue and Periodic Acid-Schiff (P.A.S.) technique in addition to metachromatic stains.

The technique of the Alcian blue-P.A.S. stain and the theory underlying it has been reviewed by Mowry (1963). Alcian blue is a copper phthalocyanin derivative which will stain with acid mucopolysaccharide substrate by an interaction which is pH dependent. At a pH above 3 it reacts more strongly with carboxyl groups than sulphate groups but when the stain is used at a pH of 1 the carboxyl groups are undisocciated and only the sulphate radicals of the acid mucopolysaccharide are able to react, producing an intense turquoise-blue coloration (Fig.15). Another important property of the dye is that, unlike other basic dyes, it has no affinity for ribonucleic acid (R.N.A.) so that there is a general absence of cytoplasmic staining.

The Periodic Acid-Schiff (P.A.S.) reaction is considered specific for the detection of neutral carbohydrates (Quintarelli, 1968). The method is based on a two step reaction. Periodic acid, a strong oxidising agent, is applied to tissue sections to liberate aldehydes from polysaccharides and the site can then be stained with Schiff reagent (basic fuchsin bleached with sulphurous acid) which is restored by the aldehydes to its unbleached magenta colour. The colour varies from pale pink to purple red

(Fig.15) and the intensity depends on the number of reacting aldehyde groups. Water soluble sugars in the tissues do not affect the reaction as these are washed out by the aqueous solutions during processing.

Toluidine blue was used as the metachromatic stain (Lillie, 1965). Metachromasia is a property exhibited by tissues containing high molecular weight polyelectrolytes, which when treated with aqueous solutions of certain dyes take on a colour different from that of the dye employed. A number of histological dyes have this property of exhibiting a "dilution shift" in that they change colour in relation to their concentration. In addition to Toluidine blue the group includes Azure A, Bismarck brown, and Safranin red O. Toluidine blue changes from its blue colour in dilute aqueous solution to reddish-purple or violet when in contact with sulphated mucopolysaccharides, including those occurring in cartilage. This colour corresponds to its concentrated dye solution and indicates either a concentration or a change in its molecular state at the point of action. These cationic dyes have the ability to bind to the negatively charged tissue polyanion sites by strong electrostatic bonds and then for metachromasia to occur there must be dye-dye interaction, (Rosenberg, 1971). This can only occur when the negatively charged polyanion sites are sufficiently close together to permit weak bonds to form between adjacent dye molecules. Thus this capacity is greater in the polyanions with short interchange distances such as heparin and chondroitin sulphate and weaker with wider distances as occur in hyaluronate. Treatment with ethyl alcohol is said to abolish

dye-dye interaction and metachromasia (Pal & Schubert, 1962). More recent work by Ramalingam and Ravindranath (1971) showed that this only occurred with the uncombined polyanions and not when they are in association with tissue proteins, so that ethanol could be used in processing the sections. The stain produces deep blue nuclei and a light blue cytoplasm in the sections with a marked red-purple to violet coloration in the cartilage matrix (Fig. 16).

The significance of the histological appearances in both control and experimental rudiments were interpreted in relation to previous studies on bone and cartilage. Bevelander & Johnson (1950) studied the distribution of alkaline phosphatase, glycogen and neutral mucopolysaccharides in relation to the process of mineralisation in developing membrane bone. Using a P.A.S. technique they showed neutral mucopolysaccharide to be present in the fibres and fibroblasts of periosteum at all stages; this later appeared in the bone matrix, staining more intensely as mineralisation proceeded.

Heller-Steinberg (1951) studied long bones from young rats using the same P.A.S. technique together with metachromatic staining for acidic mucopolysaccharides. She found the metachromasia in bone could not be related to cell function, ground substance or bone cells and that minor variations in technique may change or intensify the site of metachromasia. She did show that when slight or extensive decalcification was used in the preparation of tissue sections the metachromasia increased; as the composition of bone alters during calcification interpretation

of sites of metachromasia was necessarily obscure. She suggested that the degree of polymerisation of the ground substance corresponded to the intensity of P.A.S. staining. Resorbing bone and new bone containing bone salts stain red, osteoid without bone salt also stains red, but fully mineralised mature bone does not stain by the P.A.S. technique.

Van den Hoof (1964) studied the changing pattern of histochemical staining in the polysaccharides of the epiphyseal plate in the rat tibia from birth to adult life. He demonstrated a gradual decrease in the metachromatic staining due to acid mucopolysaccharides and an increase in the P.A.S. staining of neutral mucopolysaccharide as maturity occurred. Metachromasia was maximal in the zone of proliferating cartilage and minimal in the resting and articular cartilage. In the zone of calcification the longitudinal elements of the cell walls showed stronger P.A.S. staining than metachromasia, while the transverse partitions showed the reverse situation. He postulated a collagen stabilising function for the neutral polysaccharide, making the longitudinal partitions more resistant to metaphyseal capillary erosion. The acid mucopolysaccharides probably form complexes with collagen, rendering the latter less stable and more easily resorbed. In the zone of provisional calcification the P.A.S. positive substances were again stronger, but in all layers the differences were relative and not absolute, indicating a reciprocity between the acid and neutral polysaccharides during the dynamic changes in the growth plate.

Bernick (1971) studied the endochondral ossification in

growing rats using the Alcian blue-P.A.S. technique. He demonstrated strong staining with Alcian blue in the matrix of the proliferating zone and the proximal portion of the hypertrophic cell layer. As mineralisation proceeded in the disintegrating cells of the distal hypertrophic zone the alcianophilia was lost and the matrix became more P.A.S. positive. The diaphyseal trabeculae still contained a central core of Alcian blue staining material but this was surrounded by bone which stained intensely red with P.A.S. On the other hand when P.A.S. was applied to fully calcified bone the matrix stained poorly due to a lesser degree of polymerisation.

Organisation of the thesis.

The study of the effects of increased oxygen concentration and pressure on mouse limb bone rudiments is reported in five parts:-

Part I - describes the preliminary experiments to evaluate the variables in the organ culture method used throughout the work. The optimum conditions for in vitro growth in air were determined and used in all subsequent experiments.

Part II - describes a comparison of growth and development of mouse limb bone rudiments, in vitro and in vivo, over a six day period. The normal in vivo development of the bones in the fore and hind limbs is outlined as a basis for this comparison. As in vitro growth falls far short of that in the intact animal, the results of this comparison are important in interpreting any effects produced by varying the oxygenation of the

gaseous phase in Part III.

Part III - describes the structural changes induced in the rudiments by increasing the oxygen concentration or pressure in the gaseous phase of the tissue culture model. Rudiments were maintained in vitro for six days and the results assessed by suitable histological and histochemical staining techniques.

Part IV - describes biochemical experiments to investigate changes in the collagen metabolism of the limb bone rudiments produced by hyperoxia. The rate of collagen synthesis and degradation was determined by biochemical and radiochemical methods using tritium labelled proline incorporated in the tissue culture medium. The site of incorporation of the isotope was confirmed histologically by the use of an autoradiographic technique.

Part V - describes experiments to determine the role of lysosomal enzymes in the degradation of bone and cartilage in vitro under conditions of hyperoxia. The enzyme activity was determined biochemically in both rudiments and culture medium and compared with controls in air. The effects of hydrocortisone and epsilon-amino-caproic acid, which stabilise lysosomes and block their enzyme activity respectively, was also investigated. Their action in blocking enzyme activity was studied biochemically and histologically.

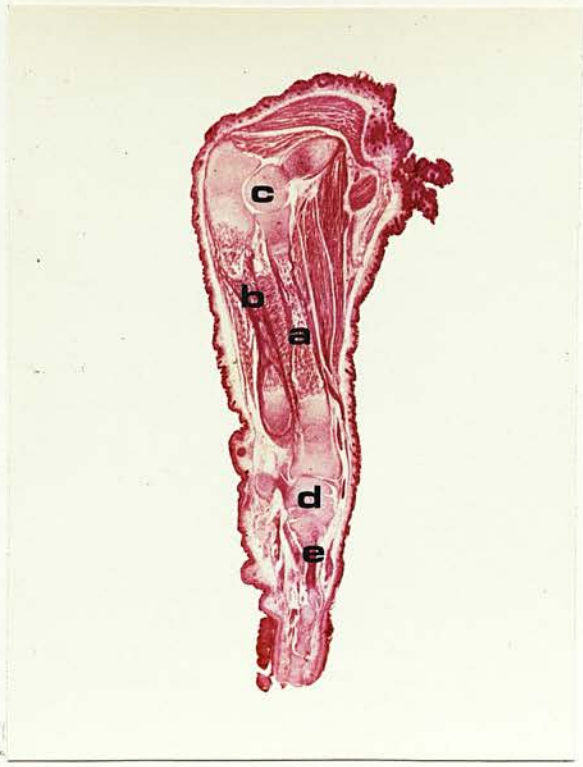


Fig. 1A Fore-limb 17-day embryo

a) Radius

b) Ulna

c) Elbow joint

d) Wrist joint

e) Carpal bones

H. & E. x 13
Longitudinal section

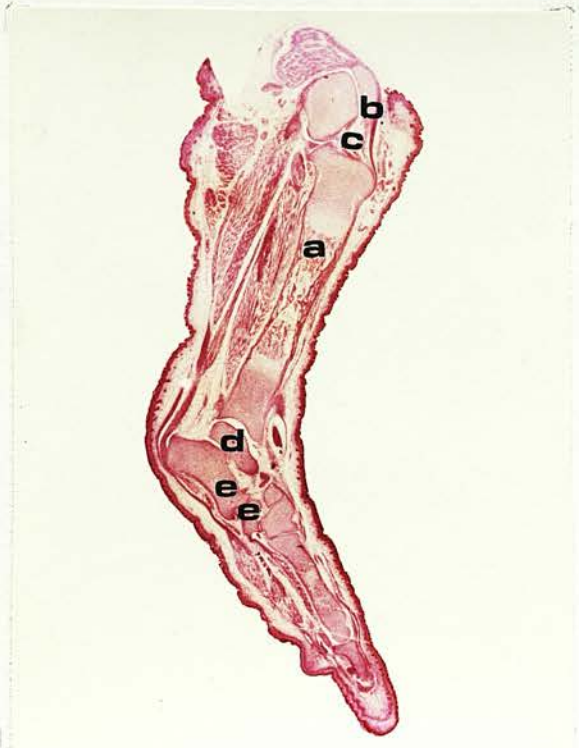


Fig. 1B Hind-limb 17-day embryo

a) Tibia

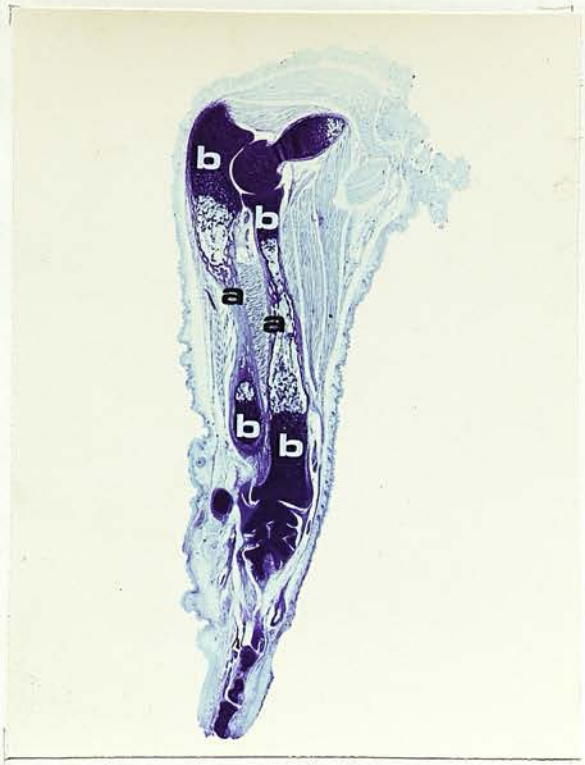
b) Patella

c) Knee joint

d) Ankle joint

e) Tarsal bones

H. & E. x 13
Longitudinal section



a) Marrow cavities

b) Cartilaginous epiphyses

Toluidine blue x 13
Longitudinal section

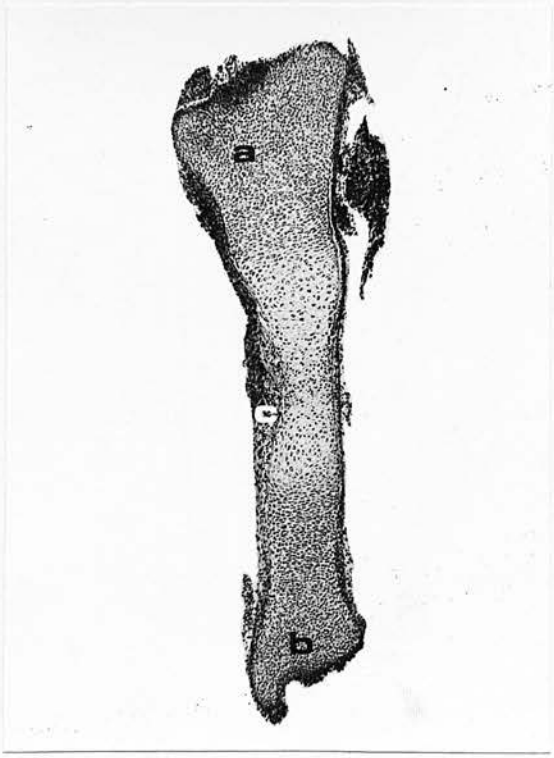
Fig. 2A Fore-limb 17-day embryo

a) Marrow cavity

b) Cartilaginous epiphyses

Toluidine blue x 13
Longitudinal section

Fig. 2B Hind-limb 17-day embryo



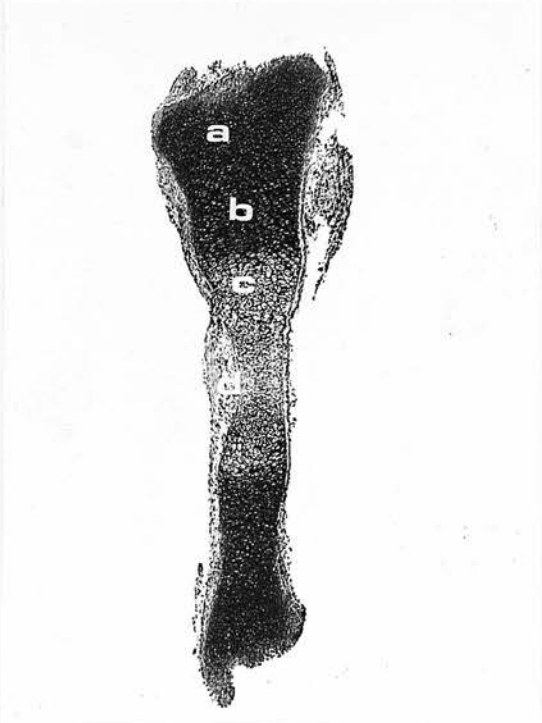
a) Proximal end

b) Distal end

c) Periosteal bone collar

H. & E. x 40
Longitudinal section

Fig. 3A Tibia 14-day embryo (early)



a) Epiphysis

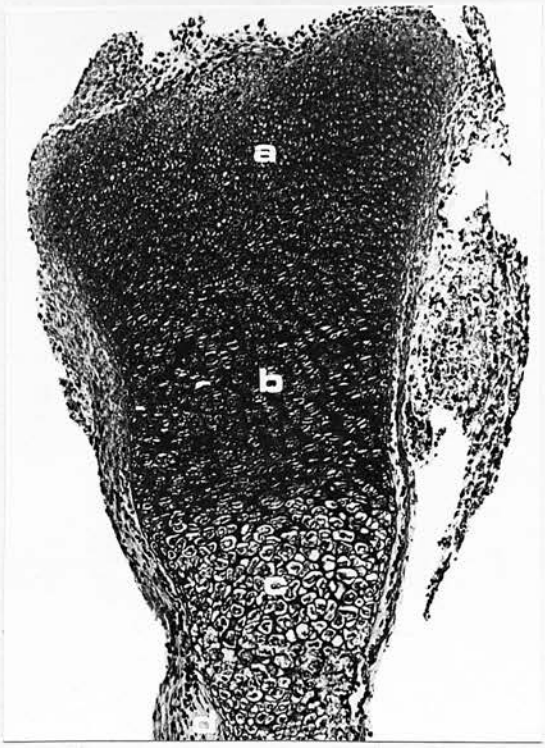
b) Proliferative zone

c) Hypertrophic zone

d) Periosteal bone collar

Toluidine blue x 40
Longitudinal section

Fig. 3B Tibia 14-day embryo (early)



a) Epiphysis

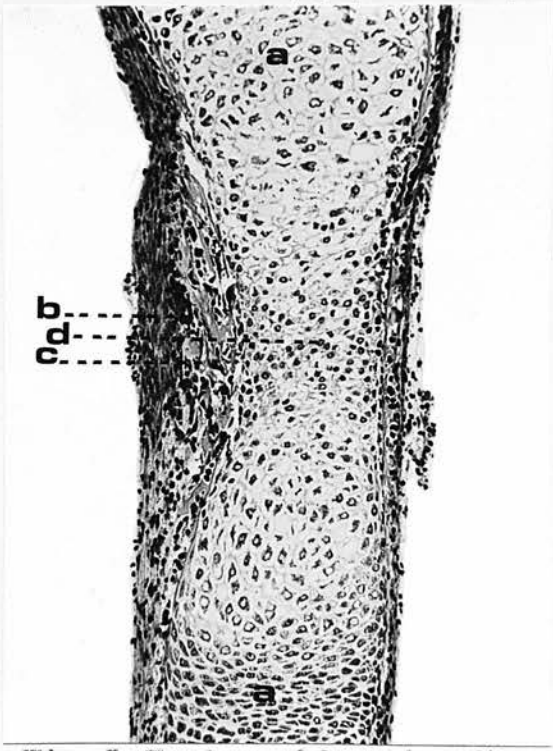
b) Proliferative zone

c) Hypertrophic zone

d) Periosteal bone collar

Toluidine blue x 110
Longitudinal section

Fig. 4 Cartilaginous epiphysis 'early' embryo



a) Hypertrophic cartilage

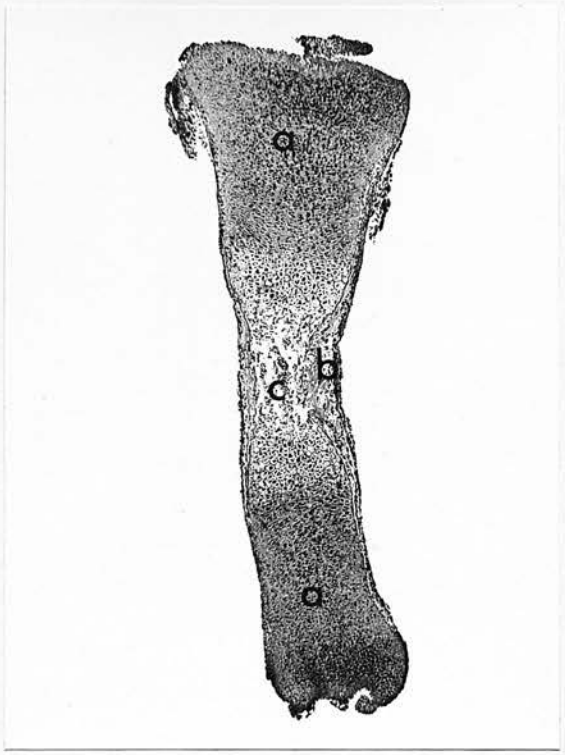
b) Osteoblastic cells

c) Periosteal bone

d) Pre-osteoblasts

H. & E. x 125
Longitudinal section

Fig. 5 Periosteal bone invading cartilaginous shaft



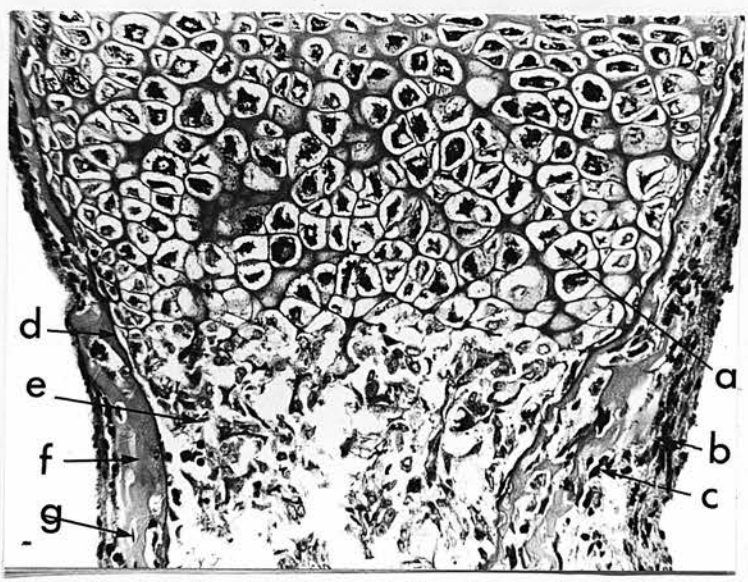
a) End cartilages

b) Bony shaft

c) Marrow cavity

Alcian Blue & P.A.S. x 40
Longitudinal section

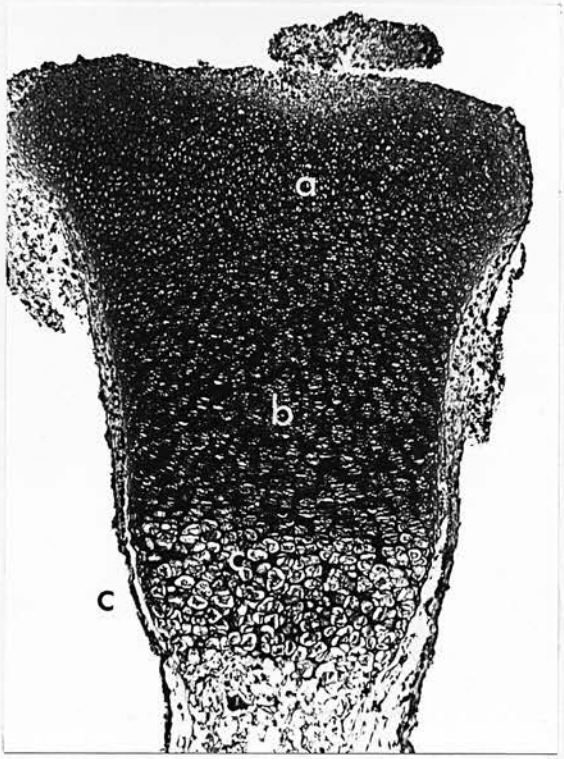
Fig. 6 Tibia 16-day embryo (intermediate)



a) Hypertrophic cells
b) Periosteum
c) Osteoblasts
d) Endosteum
e) Marrow cells
f) Osteoid
g) Osteocytes

Alcian Blue & P.A.S.
x 250
Longitudinal section

Fig. 7 Metaphysis of 16-day tibial rudiment



a) Epiphysis

b) Proliferative zone

c) Hypertrophic zone

Toluidine blue x 100
Longitudinal section

Fig. 8 End cartilage of 16-day tibial rudiment

a) End cartilages

b) Hypertrophic cell layer

c) Metaphysis

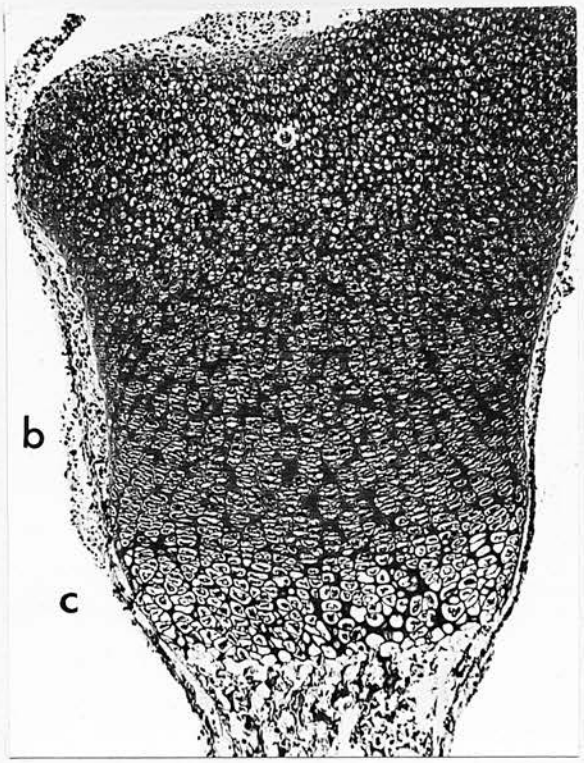
d) Bony shaft

e) Marrow cavity

Alcian blue & P.A.S. x 25

Longitudinal section

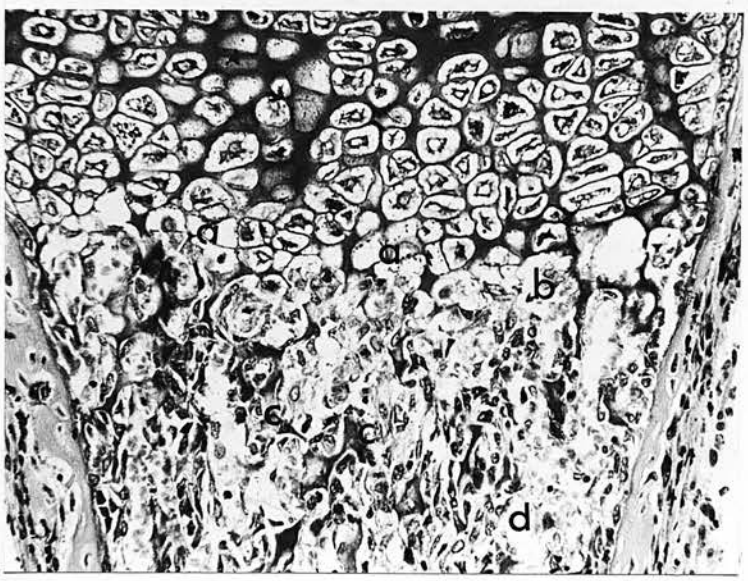
Fig. 9 Tibia 18-day embryo (late)



a) Epiphyseal zone
b) Proliferative zone
c) Hypertrophic zone

Toluidine blue x 110
Longitudinal section

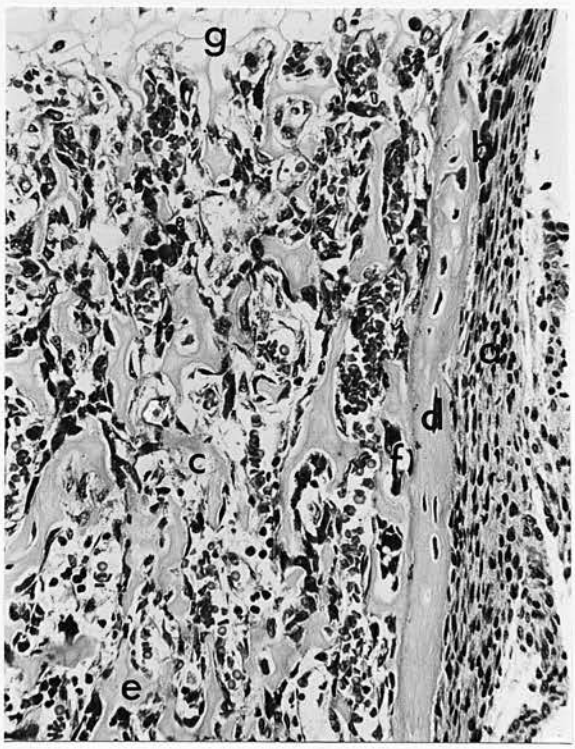
Fig. 10 End cartilage of 18-day tibial rudiment



a) Degenerate hypertrophic cells
b) Capilleries
c) Islands of degenerate cartilage with osteoid formation
d) Marrow cavity

Alcian blue & P.A.S. x 250
Longitudinal section

Fig. 11 Metaphysis of 18-day tibial rudiment



a) Periosteum
b) Osteoblasts
c) Osteoid
d) Osteocytes
e) Trabecular bone
f) Osteoclasts
g) Cartilage

Alcian blue & P.A.S. x 250
Longitudinal section

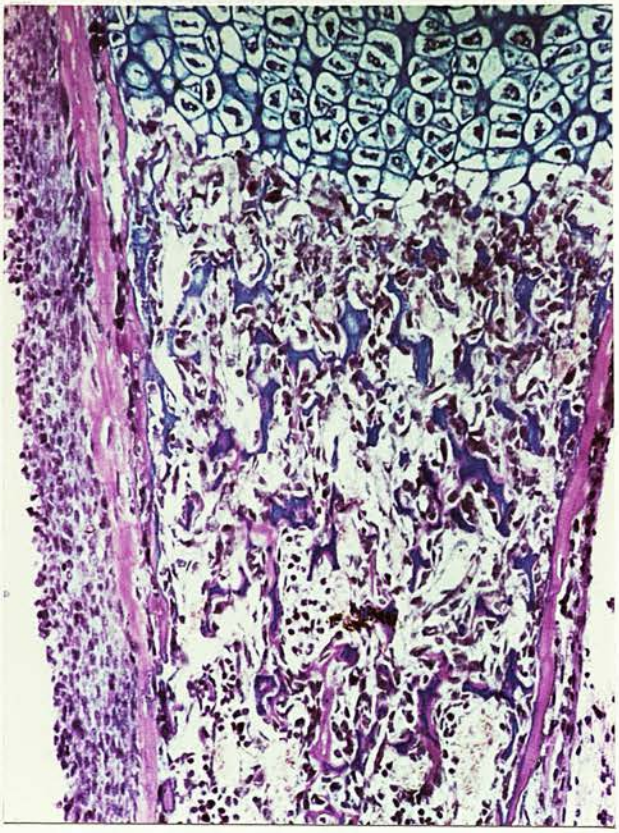
Fig. 12 Bony shaft of 18-day tibial rudiment

Longitudinal section x 175

Fig. 13 'Late' tibial rudiment - Haematoxylin & Eosin staining

Longitudinal section x 60

Fig. 14 Azan stain tibial rudiment shaft



Longitudinal section x 175

Fig. 15 Alcian blue & P.A.S. staining of tibial rudiment metaphysis

Longitudinal section x 60

Fig. 16 Toluidine blue staining in end cartilage of late tibial rudiment

PART I

EVALUATION OF THE METHODS OF IN VITRO CULTURE FOR FOETAL MOUSE LIMB BONE RUDIMENTS

INTRODUCTION

The variables in the organ culture technique used in this study are evaluated in Part I of this thesis. The experimental conditions giving optimum growth of mouse limb bone rudiments in vitro were determined and used for the remainder of the investigation.

Experiments reported on 'bone' growth in organ culture are often studies of cartilage, rather than mineralised tissue. Foetal mouse limb bones at 17 to 18 days show substantial calcified bone matrix formation in the shaft and can be used to study matrix formation and calcification, as well as cartilage growth, when cultured on a chemically defined medium. Of all the variables, the most critical factor seems to be the age of the donor embryo (Ginter, 1966; Saxen, 1966). Thus experimental series using embryos at different ages are not comparable and ideally parallel control cultures with rudiments of the same developmental age should be used, preferably the paired limb bone. In the experimental design used here each rudiment served as a control for its contralateral homologue. This technique avoids the uncertainties caused by variations between embryos and between rudiments of the same embryo.

Technique of organ culture.

The classical "watch glass" technique of Fell & Robison (1929) for the culture of embryonic skeletal rudiments utilised a plasma clot for support and nutrition of the explants. The method was later modified by Chen (1954) for use with liquid culture media by floating the rudiments on a lens-paper raft. Trowell (1954)

replaced the lens-paper with a metal grid which draws fluid up by capillary action and supports the rudiment at the liquid and gas interphase. Titanium grids were used initially but later stainless steel mesh was found equally satisfactory, providing it is carefully prepared and cleaned between cultures as recommended by Mawhinney (1968).

The culture method used throughout this study is based on that described by Fell & Weiss (1965) with a few small modifications. In this stationary technique the explants are supported on square grids of stainless steel mesh. These are contained in small disposable plastic dishes into which the culture medium is introduced so that the top of the mesh is just wetted. Two of these culture dishes are enclosed in a larger plastic Petri dish, several of which can be stacked in suitable holders and enclosed in a modified anaerobic jar. This permits accurate control of the gaseous phase by filling the jar with a suitable gas mixture. Regassing can be carried out at intervals without disturbing the culture dishes and it is unnecessary to control the atmosphere in the incubator itself. The method is simple, but fulfills the criteria for successful organ culture experiments, in that it allows accurate control of both the liquid culture medium and the gaseous phase. Direct comparison can then be made between media of differing composition, or other gas mixtures using multiple anaerobic jars. The alternative stationary culture technique described by Jones & Keeler (1971) used a Grobstein raft of millipore filter supported by a plastic ring over the depression of a glass dissecting slide containing the culture

medium. This appears to offer little advantage and is more cumbersome and expensive to use.

As an alternative to stationary cultures, some workers have used a roller tube apparatus which allows a constant flow of the liquid medium over the explant (Ito & Endo, 1956; Gaillard, 1961; Goldhaber, 1961). This seems to offer little advantage over the stationary technique and because of the immersion of the rudiment in the medium the diffusion of the gaseous phase may be variable and less accurately controlled. Schwartz (1968) described a continuous flow organ culture system for the growth of embryonic rat limb bones with very accurate control of both liquid and gas phases. This method, though ideal, would prove too expensive for use in a large number of experiments.

Choice of culture medium.

Most of the early work on the growth of cartilage and bone in vitro used the so called natural or biological media. These usually consisted of a mixture of plasma and embryo extract which was then allowed to form a clot in which the explant was incorporated. Embryo extract has a variable and unknown biological activity but was felt by many workers to be necessary for the growth and differentiation of embryonic chick limb bones in culture (Gaillard, 1935; Endo, 1960).

Synthetic or chemically defined media were developed as an alternative to the natural media and were first used by Wolff et al (1953). Their original medium was simple and consisted of a few amino-acids and agar, the growth produced being poor in comparison with plasma clot. Later workers have elaborated the

composition of synthetic media in an attempt to supply all the nutritional factors necessary for growth and differentiation, one of the most successful for the growth of cartilage and bone has been BGJ, developed by Biggers et al (1961). This gave comparable growth to natural media but after four days in culture the rudiments show a progressive hydration, particularly in the cartilage.

Most published work comparing the growth on synthetic and natural media has utilised the chick embryo long bone and not the mammalian model. Endo (1960), from a study of the histology on nine day chick femora grown in culture, concluded that embryo extract was necessary for osteoblast maintenance and osteoid formation, while serum favoured proliferation and hypertrophy of cartilage cells with calcification of the osteoid. It is uncertain whether these findings also apply to the mouse rudiment. Niven (1931) used embryo extract with a plasma clot when growing femora and tibiae from full-term foetal mice. She was able to maintain growth for 22 days with a maximum increase in length of 33%. In contrast, Fell & Mellanby (1952) using a similar mixture of plasma and embryo extract could only demonstrate a 10% increase in the length of mouse bones over a ten day period. Gaillard (1961) has used the embryonic mouse radius as his model in many experiments on skeletal physiology. He had originally described the use of 10% embryo extract for its cultivation (Gaillard, 1961), but later found this was unnecessary and reported that Hank's solution with 15% serum gave equally satisfactory results. In contrast, Jones & Keeler (1971) showed that the embryonic mouse

radius only elongated by 10-15% in Eagle's media supplemented with 10% embryo extract, as compared with a 20% increase in Eagle's medium alone. They also reported that the rudiments grown in the synthetic medium showed increased cartilage formation but poor development of periosteum and perichondrium.

Many different synthetic media have been used for the culture of bone and cartilage. The modified BGJ medium of Biggers et al (1961) has been used most extensively and Fell & Weiss (1965) reported good results when it was used for the culture of embryonic mouse limb bones. Saxen (1966) produced contrary evidence and felt that BGJ medium was inadequate for osteogenesis and calcification in vitro. Despite this, Ellis & Peart (1970) reported better growth of newborn mouse limb bones on BGJ media as compared with two other chemically defined media, T8 and TC 199. The only other comparative study was that of Zanelli et al (1969) who compared the growth and resorption of foetal mouse calvaria in BGJ, TC 199, NCTC 109, and NCTC 1066. They observed spontaneous resorption with all the media except BGJ and recommended this for biochemical studies on hormone induced resorption, although this does not indicate that it was the most suitable medium for normal development.

Most synthetic defined media are based on a balanced salt solution designed to equilibrate with a gas phase containing 5% carbon dioxide, giving a pH in the physiological range. The control of normal pH and osmotic pressure in the medium is necessary for cell survival, but may also be an important factor in stimulating bone formation. Paff (1948) grew femora from 7 day chick embryos on natural media using two different gaseous phases,

with and without carbon dioxide. He maintained the rudiments for two weeks at two pH levels; i.e. 7.0-7.3 or 7.8-8.0. His results showed that more bone is formed and more calcium is deposited at the low than the high pH. He suggested that for optimum bone formation in culture, the pH should be maintained in the direction of less, rather than more alkalinity.

Because of the conflicting evidence in the literature it was felt necessary to compare the growth and histological differentiation of late embryonic mouse limb bone rudiments on both natural and synthetic media. Experiments were also carried out to compare some of the available chemically defined or synthetic media using the same model, so that the best could be selected for the remaining part of the study. To be effective, a synthetic medium must provide good conditions for the survival of the explanted organs. Integrity of the structure of the explanted organ must be preserved, and neither its subsequent differentiation nor its functional activity must be affected. The effects of the addition or subtraction of other variables can then be studied. In the case of embryonic organs, the aim is to obtain some development of the explants in vitro as well as preserving their normal histological structure.

Use of serum supplements.

Although chemically defined media have largely replaced embryo extract as a basis for the culture of skeletal rudiments, many workers still recommend supplements of serum for optimum growth (Fell, personal communication). This introduces a

biological variable, but by heat inactivation, complement and other heat labile factors can be removed leaving the proteins for enrichment.

Endo (1960) studied the use of horse serum in varying concentration with chick embryo extract and Gey's salt solution as a medium for culture of nine-day chick embryo femora. He concluded that serum favoured the proliferation and hypertrophy of cartilage cells and was necessary for calcification of osteoid, but that the osteoid itself was not formed unless embryo extract was also present. Teaford & White (1964), again using embryonic chick femora, reported that the addition of serum to Waymouth's medium enriched with embryo extract did not improve the growth or differentiation. For the culture of mammalian rudiments, Gaillard (1961) used 15% serum to enrich his synthetic Hank's solution but did not report the growth obtained with the chemically defined medium alone. His experiments were concerned with the effects of parathyroid hormone on mouse radii in vitro and the serum was added as a carrier for the hormone. Fell & Weiss (1965) compared the growth of foetal mouse limb bones on BGJ medium, with and without 15% serum. They reported that more cellular activity, both destructive and synthetic, with the serum supplement, but the product they used was not heat inactivated. Similarly, Liskova & Jean (1970) showed an increased osteoclast cell production in rat long bones grown on TC 199 defined medium supplemented with calf serum, but did not state whether this was heat inactivated. Saxén (1966) reported that the addition of 10% serum to BGJ medium was necessary for adequate calcification,

though viability in the rudiments could be preserved without its use. Using the same BGJ medium, Ellis & Peart (1970) suggested the addition of 25% heated calf serum to give optimum growth of mouse limb bones. In contrast, Gorham & Waymouth (1965) reported that serum was not necessary for the development of perichondral bone in mouse limb bones cultured on their chemically defined medium MAB 87/3, though this included insulin as a supplement.

Because of the confusing evidence in the literature experiments were carried out to compare the growth of late embryonic mouse limb bones on chemically defined media, with and without supplements of heat inactivated serum in a concentration of 5 or 15%. Serum supplements were to be used in later experiments to estimate the release of lysosomal enzymes from rudiments into the medium during the period of culture. The serum is necessary to act as a carrier for the enzymes, to maintain their activity during the 48-hour periods between collections. To correlate the biochemical and histological findings it was necessary to observe the changes induced by the addition of serum to the chemically defined medium.

Role of Ascorbic Acid

Vitamin C or ascorbic acid is included in small amounts in most synthetic culture media, but its importance as a key growth factor has only recently been fully recognized.

Jeffrey and Martin (1966) conclusively demonstrated that the addition of 50 μgm/ml of ascorbic acid to defined medium increased the collagen content of 8-9 day chick tibiae growing

on it. This finding was confirmed by Reynolds (1966), who showed that the same dosage prevented the hydration of the end cartilages normally seen after four days' growth on the BGJ defined medium. This was accompanied by denser metachromatic staining of the mucopolysaccharide, a thicker bone collar around the shaft, and more hypertrophy of the cells in the diaphysis. Reynolds attributed the excessive hydration to a failure of collagen synthesis after four days when the endogenous stores of the vitamin became exhausted. The altered hexosamine to collagen ratio in the abnormal extracellular matrix increased its water-combining power. In later work, Reynolds (1967) reported that the vitamin action was specific and that related compounds were not as effective in promoting collagen synthesis. Iso-ascorbic acid was about one-third as active in the same dosage, while dehydroascorbic acid was quite ineffective.

The action of the vitamin seems to be mainly on collagen synthesis and Jeffrey and Martin (1966) suggested that it is concerned with the microsomal hydroxylation of proline in a collagen precursor. There is also some evidence reported by Kodicek (1965) that depletion of the vitamin interferes with mucopolysaccharide synthesis by depressing the incorporation of glucose into galactosamine.

The aerobic metabolism of chick tibiae cultured in Eagle's medium was studied by Ramp and Thornton (1968) using a roller tube technique. By estimation of oxygen consumption and lactic acid formation, they showed that the oxidation rate was linear with time and that the requirement of vitamin C for normal

aerobic bone cell metabolism was 50 μgm/ml per day in air. With higher concentrations of oxygen an increase in vitamin C content would be required.

Similar requirements probably occur in the mammalian limb bone rudiment in vitro. Jones and Keeler (1971) reported that the addition of vitamin C in a dosage of 0.2-2.0 mgm/ml, to either natural or synthetic media, gave a marked increase in both osteoid and cartilage formation in 15-day embryonic mouse radii. This effect was well maintained beyond the fourth day of culture when the normal stores of the vitamin are used up.

Most commercial synthetic media have a rather small content of vitamin C; for example the 5 μgm/ml in TC 199 (Burroughs Wellcome). These require supplements, particularly in experiments using high concentrations of oxygen in the gaseous phase. Reynolds (1966) has suggested that a minimum of 150 μgm/ml is incorporated in any culture medium and this dosage is included in the P6 modification of BGJ medium used in this study. Other experiments have been carried out to determine the histological effects of supplementing the normal levels of ascorbic acid in the TC 199 medium.

Growth of embryonic mouse limb bone rudiments in vitro.

Niven (1931) was the first to report the growth of femora and tibiae from late foetal mice on a solid plasma clot containing embryo extract. In her experiments the rudiments increased in length by up to 33% over a 22-day period. She also reported the commencing formation of secondary ossification centres in

the end cartilages. Fell & Mellanby (1952) grew late foetal and post-natal mouse limb bones on solid natural media but could only produce a 10% increase in length over a 10-day period. They observed that the periosteal bone reorganised to become less trabecular and more compact, while in the younger explants bone resorption slightly exceeded bone deposition. Despite this, few osteoblasts or osteocytes were degenerate, except in the innermost periosteal trabeculae and in the endochondral spongey bone. The endochondral ossification was almost arrested, although there was some invasion of the hypertrophic cartilage by cells from the marrow cavity with loss of mucopolysaccharide staining in the ground substance. Elsewhere in the cartilage the matrix and its metachromatic staining was well preserved, although the proliferative zone showed signs of diminished activity. Here the cells showed loss of their columnar arrangement, becoming more rounded and difficult to distinguish from the adjacent cartilage cells. Although the marrow cavity enlarged, as the hypertrophic cells were invaded, its contained haemopoietic cell population degenerated and was replaced by sparse reticulum cells and fibres.

In describing the in vitro growth of 14-16 day embryonic mouse radii, Gaillard (1961) noted little difference in the growth on synthetic as compared with natural media, but gave no details of increase in weight or length. Biggers et al (1961) were the first to culture 15-day-old embryonic mouse tibiae on a completely chemically defined medium. They reported over 100% increase in length during a 6-day culture period, far greater than in any other study, but this was not accompanied by an equivalent

increase in dry weight. The rudiments they used were mainly cartilaginous with very little periosteal bone present, so that it is probable that most of the increase in length was due to the hydration affecting rudiments grown on synthetic media. In a study using 16-day embryonic mouse ulna and radius, Saxen (1966) showed a 10.5% increase in total length over a 10-day culture period on synthetic medium enriched with 10% serum. The increase in length of the mineralized zone of the bony shaft was relatively greater, increasing by 136% over the controls. There was an associated increase in dry weight of 34% and of nitrogen content by 12%. He concluded that maximal calcification and growth occurred with 16-17 day rudiments and that it was slower in younger or older bones.

The growth of post-natal mouse long bones in vitro was described by Rajan (1969) using explants from 10-day-old mice. These were maintained in culture on a modified BGJ medium enriched with 15% serum during a 12-day period. He noted that, although the cartilage remained healthy, the bone showed increasing necrotic changes after the fourth day in culture. These changes could be partly reversed by increasing the oxygen concentration in the gaseous environment. When this was increased to 50% the survival of bone improved but it proved injurious to the cartilage which showed degeneration and resorption. Ellis & Peart (1970) used younger one-day postfoetal mouse limb bone rudiments and showed a 22% increase in length over a six-day period of growth on BGJ medium enriched with 25% serum. They reported good preservation of the anatomy with both periosteal and endosteal bone formation,

though there was no growth by endochondral ossification.

The two major problems in growing mammalian bones in vitro are the failure of normal mineralization in newly-formed osteoid and the arrest of endochondral bone formation. The only report of normal endochondral bone growth came from the experiments of Crelin (1967) and Crelin and Koch (1967). They used a synthetic Eagle's medium, supplemented with 10% horse serum and 3% embryo extract to grow embryonic mouse pubic bones. The rudiments were labelled by exposure to tritiated thymidine and the cells studied by autoradiography through the developmental stages of periosteal and endochondral bone formation. They demonstrated that the hypertrophic chondrocytes of the calcified cartilage could survive dissolution of their matrix and transform into the osteoblasts and osteocytes, which formed endochondral bone even in the absence of blood vessels.

In selecting the most suitable age of limb bone rudiment for optimum in vitro growth the findings of Ginter (1966) are very relevant. He compared the growth over a six-day period of long bones from mouse embryos of 13, 14 and 15 days development. In common with other workers, he noted the most active growth in the first two days in culture and that all significant elongation had occurred by the fourth day. There was little difference to be found in the rudiments at four or six days. Most of the growth occurred in the cartilage by hypertrophy of chondrocytes and an increase in the amount of ground substance between the cells. Periosteal bone, when present, continued to thicken and the first stage of endochondral ossification by destruction of the

hypertrophic cartilage cell layer was noted. He concluded that both in culture and in vivo the most highly differentiated anlagen of the bone showed the smallest increase in length and that this difference in the relative increase in the length of the anlagen persisted. Unfortunately he used a complex mixture of a chemically defined TC 199 medium, with natural supplements of serum and embryo extract and it is uncertain whether these observations are true of chemically defined medium alone.

Experimental evaluation of tissue culture variables.

Because of the confusing evidence in the literature reviewed, experiments were designed using a stationary culture technique to evaluate the following:-

(1) To compare growth and viability on synthetic and natural media.

(2) To compare growth and viability on three different synthetic media; TC 199, Eagle's basal medium, and BGJ (P6 modification).

(3) To determine the influence of serum supplements in promoting bone formation or resorption in culture.

(4) To determine the effect of varying ascorbic acid dosage on bone production and the prevention of cartilage hydration.

(5) To investigate the variation in growth of rudiments obtained from embryos of different ages.

MATERIALS AND METHODS

(1) Mice

A commercial strain of mice, T.O. Tuck's No.1 (A. Tuck & Son Ltd., Rayleigh, Essex) was used throughout this work. Initially pregnant females were obtained from the suppliers, but some difficulty was experienced in accurate timing of the gestation period which did not always correspond with that stated. In later experiments surviving male and female offspring were used for further breeding in the laboratory by the following method.

Monogamous pairs were placed in cages in the early afternoon and left overnight. The females were examined each morning for the presence of vaginal plugs and if these were present the male was removed. The day of appearance of the plug was taken as day 0 in the gestation period of the embryo. About 75% of the mice with plugs proved to be pregnant.

The gestation period with this strain of mouse was 19 days with an average litter size of 11 embryos.

(2) Isolation of bone rudiments

Embryos ranging in age from 14 to 19 days were used in these experiments. Pregnant females were killed by cervical dislocation and the embryos removed aseptically, using fresh sterile instruments for each stage of the dissection. After cleaning with 70% alcohol, the skin of the abdomen was opened vertically with scissors and stripped back laterally to expose the peritoneum. This was moistened with 70% alcohol

and incised in the midline to expose the uterine horns with the contained embryos. The gravid uterus and its contents were removed and immersed in Hank's balanced salt solution (Burroughs Wellcome) in a sterile covered petri dish. This solution, of composition shown in Appendix B, provides a physiological pH in the range 7.3-7.6 when equilibrated with air and was used at room temperature for all stages of the dissection. The uterus was opened and the embryos separated from their foetal membranes and placenta before transfer to another dish of Hanks' solution. Biggers (1960) stressed the importance of early separation of the embryos from the uterine membranes if maximal elongation of rudiments is to be obtained.

Fine watchmaker's forceps and ophthalmic scissors were used to remove the fore-limbs and hind-limbs from each embryo, which were then placed in pairs into Hanks' solution in the recess of a sterile dissecting slide (Hospital and Laboratory Supplies Ltd.). Each dissecting slide was enclosed in a sterile petri dish and kept in the incubator until required.

Dissection of the limbs was carried out under magnification with a Wild M5 stereo-microscope using cataract knives and watchmaker's forceps. The skin and soft tissues were cut away from the rudiments, which were disarticulated at their proximal and distal joints, with care not to detach the periosteum. During dissection the rudiments were moistened at intervals with fresh Hanks' solution from a Pasteur pipette. When clean, the rudiments were transferred with

forceps to the grids in the prepared culture dishes. The tips of the instruments were resterilised in a beaker of boiling distilled water between dissections. A sterile museum jar placed on its side was used to hold the tips of instruments and pipettes when not in use. All glassware and instruments were cleaned prior to use and sterilised by dry heat at 160°C for two hours.

(3) Culture technique

(a) Preparation of culture dishes.

The culture dishes were prepared prior to isolation of the rudiments and were kept in an incubator at 37.5°C until required. Using a sterile non-touch technique, two 30 mm disposable plastic petri dishes were placed inside a 10 cm diameter plastic petri dish carpeted with three thicknesses of No.1 Whatman's filter paper. The lids were removed from the small dishes and discarded. Both sizes of plastic dish were supplied pre-sterilised by gamma-irradiation (Falcon Plastics Ltd.). A grid of stainless steel mesh, 2 cm square with two opposite edges folded down to form 2 mm legs, was placed in each small dish. The grids were cut out from a roll of 'Micromesh' expanded stainless steel, F.D.P. quality No.978/mm (The Expanded Metal Co.). Prior to use they were washed in concentrated nitric acid, running tap water, several changes of distilled water, and 70% alcohol, before sterilisation by dry heat at 160°C for two hours.

The filter paper carpet in the large petri dish

was moistened with 5 ml of sterile normal saline introduced by pipette to produce a moist atmosphere. The selected culture medium was added to each small dish using a disposable Pasteur pipette. A volume of 1.5 ml was found to be required to just wet the top of the grid by capillary action. To prevent the formation of trapped air bubbles beneath the grid, the first five drops were placed on its surface and the remainder introduced into the dish against the closed side of the grid. The use of millipore filter or lens paper on the surface of the grid was shown to be unnecessary by Mawhinney (1968) and might even inhibit rudiment growth. All manipulations were carried out under a perspex shield to prevent contamination.

The plastic petri dish containing the small culture dishes and grids is illustrated in Figure 17. A diagrammatic representation of the complete culture system with the rudiments on the grids is shown in Figure 18.

(b) Gassing of cultures.

When prepared the petri dishes were stacked in a stainless steel rack which fits inside a modified MacIntosh-Fildes anaerobic jar (Fig.19). The jar had its side outlet tube blocked off but could be gassed through the inlet and outlet valves on the lid after closure. The gaseous phase used in these experiments was 95% air and 5% carbon dioxide, supplied in 120 cubic

ft cylinders to an accuracy of ± 0.1% (British Oxygen Co., Special Gases Division). The gas was passed through a reducing valve and delivered by sterile tubing to the anaerobic jars while these were contained in an incubator at 37.5°C. A flow rate of 2 litres per minute was used for ten minutes to flush out the jars prior to closure of the outlet and inlet valves.

(c) Regassing and change of media.

The jars were regassed every 24 hours during the period of in vitro culture using the same technique. The evaporation from the saline soaked filter paper in the petri dishes provided a sufficiently moist atmosphere without the need to humidify the gas itself.

The culture medium was changed every 48 hours with new medium of known pH previously warmed to 37.5°C in the incubator. Using sterile Pasteur pipettes the old medium was sucked off from each dish and its pH measured prior to discard. The new medium was added to each dish using a fresh pipette with care to avoid trapping air bubbles beneath the grid. The explants were then turned over through 180° on the grid surface using sterile dissecting needles. This prevents the formation of cellular outgrowths which sometimes anchor the bone rudiments causing distorted growth and bending. The culture dishes were replaced in the jar and regassed in the incubator as described previously.

(4) Culture Media

Synthetic culture media of three types were used in the

experiments to determine optimum conditions for growth in rudiments. These were T.C.199 (Burroughs Wellcome), P6 modification of BGJ medium (Strangeways Laboratory, Cambridge) and Eagle's Basal Medium (Burroughs Wellcome). In experiments to determine the effects of other variables the media were supplemented with heat inactivated foetal calf serum (Burroughs Wellcome) in 5% or 10% concentrations, and vitamin C (B.D.H.) in concentrations up to 150 gm per ml.

(a) T.C.199 medium - was supplied in powder form with the chemical composition shown in Appendix B. It was made up in one litre batches by dissolving 10 g. of powder in 1000 ml of de-ionised water with the addition of 200,000 units of penicillin and 100,000μgm of streptomycin. The solution was passed through a 47 mm membrane 'Sterilin' filter (Millipore Ltd.), with pore size of 0.22μ, and stored frozen in sterile glass bottles in 100 ml aliquots until required. One bottle was sufficient for each set of experiments and after thawing 5 ml of 4.4% sodium bicarbonate solution was added. Additions of serum and vitamin C were made to the desired concentration before final millipore filtration into 1.5 ml aliquots. This volume was sufficient for one culture dish and was stored in disposable plastic screw capped vials (Sterilin Ltd.) at $0^{o}C$.

(b) BGJ (P6 modification) medium - was supplied as a complete medium in dried form with the composition shown in Appendix B. It contained antibiotics, sodium bicarbonate,

and 15 mg./100 ml. of vitamin C requiring only to be dissolved in double distilled water in a concentration of 16.38 g. in 1000 ml. After bulk filtration the medium was stored frozen in 100 ml. aliquots. One bottle was thawed for each batch of experiments and after addition of the appropriate serum concentration was refrozen in 1.5 ml. volumes until required for use.

(c) Eagle's Basal Medium - is a simpler culture medium consisting of a balanced salt solution with only a few essential amino-acids and vitamins. It was supplied in a 10x concentrate sslution in 100 ml. amounts. A single strength solution was made up in one litre batches by adding 100 ml. of concentrate to 900 ml. of de-ionised water, together with 200,000 units of penicillin and 100,000μg. of streptomycin. After membrane filtration this was stored frozen in 100 ml. aliquots. One bottle was thawed to supply sufficient medium for one batch of experiments by the addition of 5 ml. of 4.4% sodium bicarbonate solution and the appropriate concentration of vitamin C and serum. This was dispensed in 1.5 ml. aliquots and frozen until required for use.

(d) Chick embryo extract (Flow Laboratories) - was used in a few experiments to provide a natural medium for comparison with a synthetic chemical defined medium. It was supplied in a sterile liquid form in 20 ml. vials. The contents of one vial were added to 80 ml. of single strength TC 199 medium. The resultant

mixture was stood for 30 minutes in a water bath at 37°C to obtain maximum solution of the nutrients. The insoluble residue was removed by centrifugation at 3000 r.p.m. for 10 minutes. Tritiated proline, 1.0 µCi/ml., was added to the supernatent, which was stored frozen in 1.5 ml. aliquots. The medium when used thus contained 20% embryo extract with a radioactive collagen label.

(5) Measurement of growth

(a) Growth in length.

The overall length of each rudiment was measured at the time of explantation using an eyepiece graticule with a millimetre scale. Repeat measurements were made at the time of each media change and at the completion of the culture period to determine any change in length.

(b) Weight of rudiments.

In some experiments rudiments were weighed collectively at the end of the culture period and compared with the contralateral rudiments from the same animals used as zero controls or experimental controls. It was not possible to weigh experimental rudiments at the time of explantation because of the problem of maintaining sterility.

(6) Histological Examination

(a) Fixation.

At the end of the culture period rudiments were fixed routinely in Zenker's solution, containing 5%

acetic acid, for 40 minutes and then transferred to tap water. In the few experiments where rudiments were to be examined by autoradiography the fixative was changed to Bouin's solution, which does not contain heavy metal salts.

(b) Dehydration and embedding.

The rudiments were dehydrated by passage through graded alcohols up to absolute. To facilitate orientation of the small bones at the time of embedding these were lightly stained by adding 5% eosin to the 98% alcohol. After dehydration the rudiments were cleared in chloroform prior to embedding in Paraplast paraffin wax (Sherwood Medical Industries) with a melting point of 56-57^{o}C. A double technique was used with vacuum impregnation followed by embedding in a disposable plastic mould (Tissue Tek).

(c) Sectioning.

After trimming blocks were mounted and cut at 5μ on a Jung rotary microtome to give serial longitudinal sections. Three or four consecutive sections were placed on each glass slide for staining or autoradiography.

(d) Autoradiography.

In rudiments which had been prelabelled with tritiated proline, some sections were used for autoradiography by the dipping technique. Sections were first deparaffinised with xylene and then rehydrated by passage down through

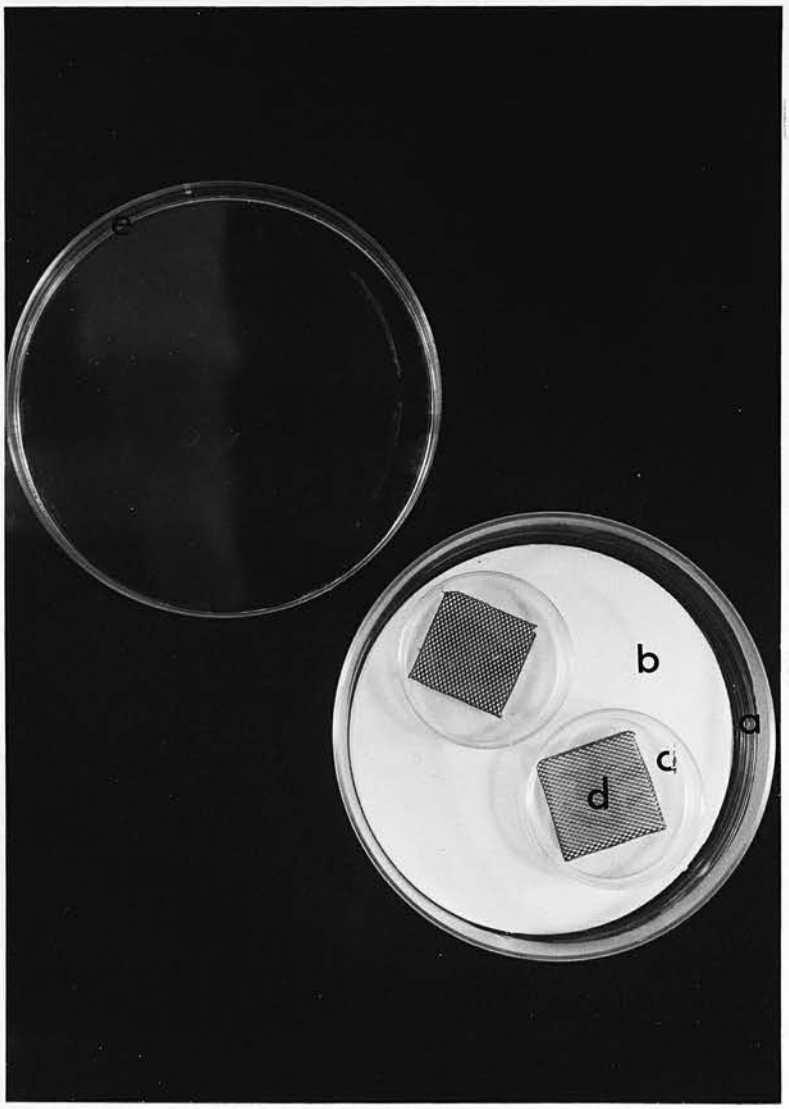
graded alcohols. Sufficient nuclear emulsion, NTB3 (Kodak England Ltd.) or K2 (Ilford Ltd.), was melted in a water bath at 40°C and diluted 50% with water under dark room conditions. After stirring to remove air bubbles a slide dipping jar was filled with emulsion and the slides dipped in turn. Each slide was immersed vertically for 10 seconds and the excess blotted from its end with a gauze pad. The thin uniform layer of emulsion produced was allowed to gel in the horizontal position on drying racks. Slides were then placed in light tight boxes, sealed and exposed for four weeks at 4°C in a refrigerator compartment. All the dark room manipulations were carried out using a Kodak Wratten Series 1 safelight.

The autoradiographs were developed by immersion in Amidol developer for two minutes, rinsed in distilled water, and fixed in acid fixer solution for three minutes (all chemicals Kodak Ltd.). After the slides were washed thoroughly in distilled water they were stained prior to examination.

(e) Staining.

Sections were stained routinely with haematoxylin and eosin, toluidine blue, alcian blue and P.A.S., and Heidenhain's azan stain.

Slides used for autoradiography were post-stained after development with either Harris haematoxylin, or methylene blue-azure A-basic fuchsin (Belanger, 1961).



a) Petri dish
b) Filter paper
c) Culture dish
d) Grid
e) Cover

Fig. 17 Culture Dishes and Grids

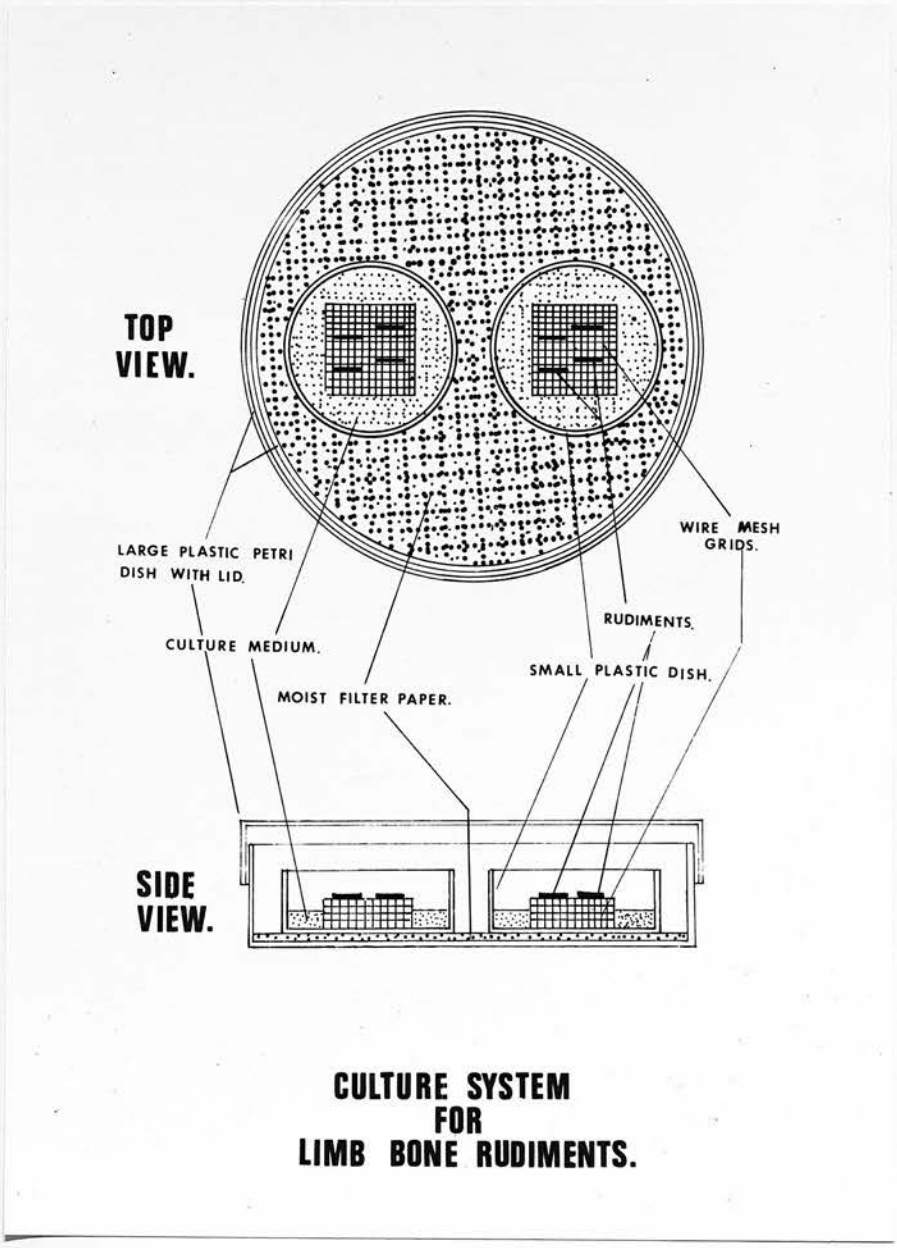
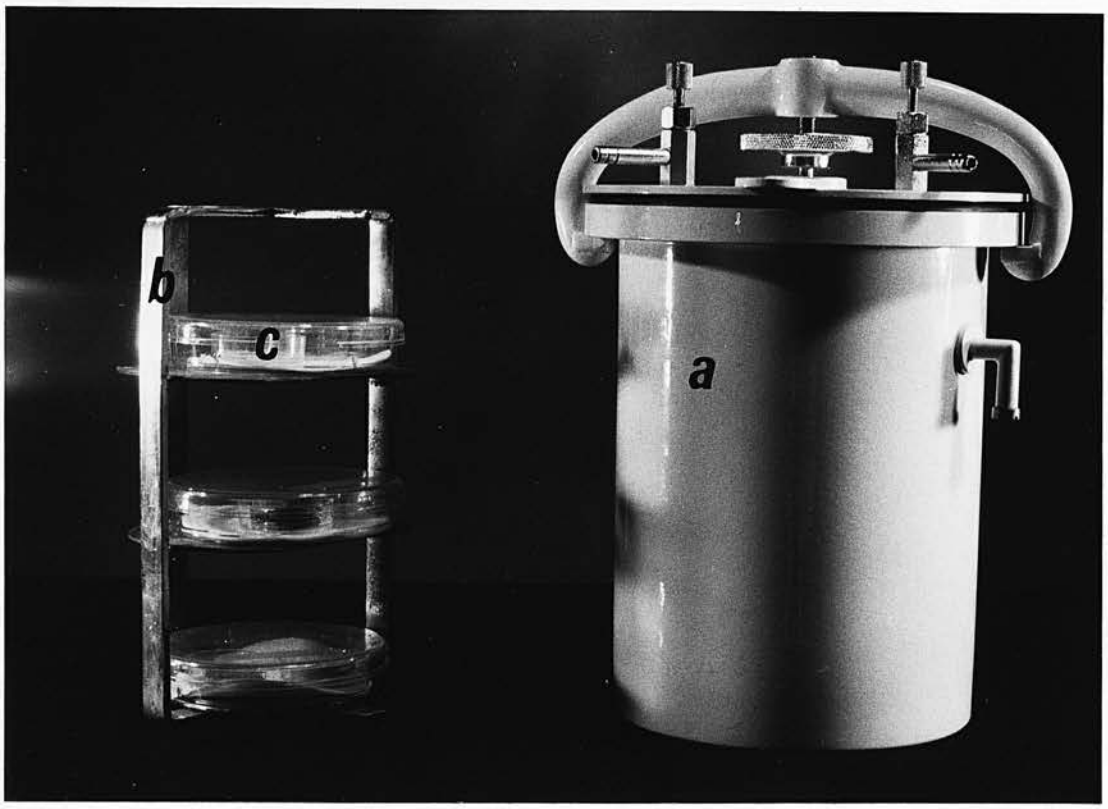


Fig. 18 Diagram of Culture System



a) Anaerobic jar

b) Steel rack

c) Petri dishes

Fig. 19 Modified anaerobic jar and petri dish rack

RESULTS

1) Comparison of Synthetic and Natural Media

Two separate experiments with six pairs of late 18-day tibial rudiments were used to compare synthetic TC-199 medium with the same medium supplemented by 20% chick embryo extract. Two pairs of rudiments were harvested at each media change. Over the 6-day period of culture the pH of both media remained within physiological limits and did not differ by more than ± 0.05. The results obtained were as follows:-

a) Increase in length.

The percentage increase in length of the paired rudiments for each 2-day period in culture is shown graphically in Figure 20. With the exception of one pair, elongation was slightly greater in rudiments grown on medium containing embryo extract. Most growth occurred during the first 2 days and any further increase in length at 4 days was usually lost by 6 days due to bending of the bones.

b) Histology and autoradiography.

(i) Embryo extract medium - after 2 days in culture the end cartilages showed evidence of central lysis, particularly in the zone of proliferation. (Fig.21A). At the cartilage centre there was loss or pyknosis of cell nuclei, vacuolation of the surrounding cells, diminished matrix metachromasia, and less glycogen staining in the cytoplasm.

Another marked feature was the thickening of the perichondrium particularly overlying the site of central necrosis. Here it

was up to 10 cells thick, lacking metachromasia of the ground substance but showing positive azan staining suggesting new collagen formation. The autoradiographs showed some generalised labelling throughout the matrix and cells of the cartilage, but this was not as heavy as activity in the cells of the thickened perichondrium (Fig.22A). At 4 days and 6 days the degeneration of the end cartilages became more marked, with widespread vacuolation, nuclear death in all layers and further loss of metachromasia. The perichondrial thickening became less marked, though exceeding that in the paired rudiments on synthetic medium.

In the bone of the shaft the periosteal layer showed thickening at 2 days in continuity with the perichondrium. On the surface the cells were more flattened but there were well marked rounded osteoblasts in the deeper layers forming new osteoid on the surface of the bony shaft (Fig.23A).There was little evidence of endochondral bone formation and some resorption had occurred in the hypertrophic zone of the cartilage. Endosteal bone formation was also arrested with some loss of osteoblasts and osteocytes in the deeper layers of the shaft bone. The cellular density of the marrow was decreased, although a few red blood cells were still present. The majority of the population consisted of small round cells of macrophages and only occasional multinucleate osteoclasts were seen. After 4 and 6 days in culture the periosteum became thin, but still contained active osteoblastic cells mainly confined to the superficial layer. Autoradiography

showed some label incorporated in the cells and adjacent matrix of the superficial periosteal layer. This persisted for up to 6 days in culture, though little appeared in the deeper matrix of the cortical bone. In the later stages of culture, grains were seen in the osteoclastic cells which were now in evidence in the endochondral region, where they engulfed the remains of the hypertrophic cartilage cells.

(ii) Synthetic TC-199 medium - The cartilage at 2 days did not show much evidence of central lysis (Fig.21B) nor was there such marked thickening of the perichondrium. The autoradiograph showed rather more label in the matrix and cells of the cartilage, particularly over the nuclei in the proliferative zone though again the label was heavier in the perichondrium, (Fig.22B). By contrast with the natural medium, the end cartilages at 4 and 6 days showed some preservation of the chondrocytes with zoning into the flattened proliferative and hypertrophic layers. Some of the more central cells in the proliferative zone showed increased vacuolation, resembling hypertrophic cells, but overall the matrix metachromasia was better preserved.

In contrast, the bone of the shaft showed more degenerative changes than the rudiments grown on natural medium. At 2 days osteoblasts were still present and active in the periosteum and between the trabeculae of the bony shaft (Fig.23B), though endochondral ossification seemed to be arrested. Few osteoclasts were present at this stage and red cells were still

present in the marrow. At 4 and 6 days the bone of the shaft became buckled on sectioning, with an associated loss of collagen on azan staining. Few osteocytes or osteoblasts remained in the deeper portion of the cortex or on its endosteal surface, while those in the superficial layers became more flattened and spindle-shaped. Autoradiographs showed little evidence of new label incorporation at the periosteal surface, though there was better preservation of the heavy labelling previously incorporated in the deeper layers of the shaft bone (Fig.24B). Some attempt at endochondral formation was also present in these later stages as shown by weak PAS positive staining of osteoid on the surface of the cartilage cores, with associated incorporation of tritiated proline label in autoradiographs.

2. Comparison of Synthetic Media.

The growth and histological viability of embryonic limb bones were compared on different synthetic media in two experiments, each using eight late tibial rudiments. The TC-199 medium was compared with Eagle's basal medium in one and with the modified BGJ medium in the other.

The results were:-

a) Increase in length.

The percentage increase in length for each set of paired rudiments during the 6 day period of culture is shown graphically in Figures 25 and 26. In both experiments the poorest growth was obtained with TC-199, although the relative difference was

much greater with BGJ than Eagle's medium. Even on BGJ medium the maximum elongation obtained was only just over 10%.

b) Histology.

(i) TC-199 and Eagle's basal medium.

There was little difference in the paired rudiments after the 1 day in culture, both showing some hypertrophy of their end cartilages. This was accompanied by early degenerative changes in some of the more central chondrocytes, narrowing and resorption in the hypertrophic cell layer, and some increase in the proliferative cell layer. The bony shaft and marrow were better preserved on Eagle's medium, although some loss of collagen on azan staining was evident in both.

After 2 days the end cartilages were more soft and degenerate showing distortion in the sections and more widespread chondrocyte death, though ground substance metachromasia was well preserved. More osteoblasts were dead on the periosteal surface of the rudiment on TC-199, while the marrow cells were replaced by a population of small round cells.

At 4 days there were two definite and distinct histological features observed, particularly in the rudiments on Eagle's medium. In the end cartilages a junctional zone developed between the proliferative and hypertrophic cell layers, while in the bone of the shaft osteoblastic activity re-appeared in the cells of the deeper layers of the periosteum. The new zone in the cartilage consisted of flattened cells invading in from the perichondrium and

showed positive PAS and azan staining. (Fig.27). At the same time the central chondrocytes of the epiphyseal zones showed increased vacuolation and PAS staining.

After 6 days both rudiments showed gross degenerative changes, particularly with TC-199. Despite the re-appearance of cells resembling pre-osteoblasts in the shaft, the bone showed increasing resorption and marrow degeneration. The changes seen in the end cartilages at 4 days were still present but in both rudiments there was a loss of metachromasia around the periphery. This was associated with the formation of a layer of fibroblasts sealing off the end cartilages from the shaft. (Fig. 28).

(ii) TC-199 and BGJ medium.

After 1 day the rudiment on BGJ medium showed definite proliferation of the end cartilages with widening of the hypertrophic cell layer, unlike the early degenerative changes seen on TC-199.

However at 2 days the hypertrophic cell layer on both media became sealed off from the shaft by the ingrowth of flattened cells showing positive PAS and azan staining.

After 4 days the central chondrocytes showed lysis on TC-199, but were vacuolated with increased PAS positive staining on BGJ.On both media the bone and marrow of the shaft showed marked degenerative changes with resorption and cell death. The periosteal bone was better preserved on

TC-199, while the trabecular bone in the zone of endochondral ossification showed less resorption on BGJ medium. Little difference was seen in the rudiments after 6 days in culture.

3. Influence of Serum Supplements

Two experiments were performed to determine the effect of adding heat inactivated foetal calf serum in a concentration of 5 or 15% to the synthetic media BGJ and TC-199. Sixteen paired late tibial rudiments were used in each experiment to compare the growth in length, histological appearance, and autoradiographic localisation of ^{3}H-proline on plain media with serum supplements. The results were:-

a) Increase in length.

The percentage increase in length of the paired rudiments for the six day period in culture is shown graphically for the BGJ medium in Figure 29 and for TC-199 in Figure 30. On BGJ medium, serum supplements gave improved growth in most pairs and the effect was more immediate and better sustained with 15% than 5%. A similar effect was seen with serum supplementation of TC-199, though the increase in length was not so great or so immediate, as with BGJ.

b) Histology and autoradiography.

(i) BGJ medium.

After 1 day in culture the end cartilages showed very little difference on the serum enriched media when compared with the plain, except for a more marked widening of the proliferative cell layer due to chondrocyte enlargement and

TABLE 2

Effect on % growth in length of Serum supplements with TC-199.

Time	Medium	% increase in paired rudiments							
1 day	TC-199	3.4	1.1	1.1	1.1	1.1	1.2	1.1	4.4
	+ 5% serum	5.6	2.2	1.1	2.2				
	+ 15% serum					4.4	2.0	1.1	7.1
2 days	TC-199		1.1	1.1	2.2		2.4	2.3	6.6
	+ 5% serum		3.3	1.1	2.2				
	+ 15% serum						2.0	1.1	7.1
4 days	TC-199			2.3	2.2		4.6		1.1
	+ 5% serum			2.3	4.4				
	+ 15% serum						4.4		4.6
6 days	TC-199				2.2				4.4
	+ 5% serum				4.4				
	+ 15% serum								9.9

TABLE 3

Effect on % growth in length of Serum supplements with BGJ

Time	Medium	% increase in paired rudiments							
1 day	BGJ	7.6	7.2	11.9	5.6	8.7	8.7	8.5	6.3
	+ 5% serum	8.3	7.2	15.0	5.4				
	+ 15% serum					10.7	10.7	13.8	12.6
2 days	BGJ		10.3	14.1	8.9		13.0	9.6	8.3
	+ 5% serum		11.3	20.4	6.5				
	+ 15% serum						15.1	12.9	12.6
4 days	BGJ			11.3	11.1			14.9	9.4
	+ 5% serum			19.4	8.7				
	+ 15% serum							16.1	15.0
6 days	BGJ				8.9				10.4
	+ 5% serum				6.5				
	+ 15% serum								16.1

division. At 2 days the cartilage on the enriched media showed less central lysis and widespread chondrocyte vacuolation than the controls. By 4 days the invasion of flattened cells from the thickened perichondrium into the proximal part of the hypertrophic cell layer was much more complete with the serum enriched media. At 6 days this divided the hypertrophic cells into two zones with the flattened cells between showing well marked positive staining with P.A.S. and azan, suggesting the presence of glycogen and new collagen (Fig.31).

In the bone of the shaft the addition of serum produced differences visible after only 1 day in culture.The endosteal resorption of bone was more marked and accompanied by the presence of numerous multinucleate osteoclasts (Fig 32), not seen with plain medium. This change was more marked at 2 days, though slightly compensated by the thickening of periosteum in continuity with the perichondrium at the bone ends. After 4 days there were very few viable periosteal osteoblasts with the plain medium, though these were still actively producing new osteoid over the junction of shaft bone and end cartilages on the enriched medium.

The tritiated proline autoradiographs confirmed that the formation of new osteoid by the periosteal osteoblasts and its resorption by macrophages on the endosteal surface was enhanced by the addition of serum (Figs.33A & 33B). After 4 days the label was no longer present in the periosteum but was still deposited in the thickened perichondrium overlying

the junction of the proliferative and hypertrophic zones of the cartilage (Fig. 34). This occurred with both plain and enriched media, but with the latter label was also deposited heavily at the site of invasion in the hypertrophic zone (Fig.35).

The changes in cartilage and bone induced by the addition of serum occurred with both concentrations, although preservation of normal morphology was marginally improved with the higher concentration of 15%.

(ii) TC-199 medium.

Differences were apparent in the rudiments grown on serum enriched media after only 1 day in culture. In the end cartilages there was less resorption of the distal part of the hypertrophic cell layer than with plain medium, though the cells at the centre of the proliferative zone were rounder and more vacuolated. The most marked feature was the thickening of the periosteal and perichondral cell layers, occurring with both 5% and 15% serum.

After 2 days in culture this thickening was even more marked, particularly towards the ends of the rudiment where it was 6 - 8 cells thick (Fig. 36A and 36B). Although some deeper osteocytes in the shaft bone were dead, as shown by the empty lacunae, the surface layer showed healthy osteoblasts and pre-osteoblasts laying down new osteoid.

After 4 and 6 days the shaft bones showed rather more degenerative changes, even with serum supplements, but although some of the deeper bone was resorbed the surface layers remained

healthy without much further thickening. No osteoclasts were seen in relation to the resorption of the deep bone, although this had been a feature with the addition of serum to BGJ medium. The end cartilages showed more cellular invasion from the thickened perichondrium into the proximal part of the hypertrophic zone with serum supplements. There was a loss of metachromatic staining and the formation of collagen at the site of invasion and in one rudiment a transverse split in the cartilage tissue appeared. As with medium BGJ little difference was noted between the addition of 5% or 15% serum.

4. Effect of varying Ascorbic Acid concentration.

As the P6 modification of BGJ medium contains a high ascorbic acid concentration of 150 µgm/ml. the experiments to determine its effect were carried out with TC-199. Pairs of late tibial rudiments were grown on the plain medium, or medium supplemented with ascorbic acid to the level of 50 or 150 µgm/ml. The results were unsatisfactory because of the inability of the bicarbonate buffer system to maintain the media with high ascorbic acid concentrations within physiological limits. When this pH was checked at each medium change it was found to differ more widely than the ± 0.1 obtained in all other experiments. In the media containing ascorbic supplements this was between 0.25 and 0.35 higher than the plain medium. The results recorded were:-

a) Increase in length.

There were no significant differences in the percentage

increases in length of the rudiments during the six days in culture. In none of the rudiments was this greater than 10% and in most cases the rudiment grown on plain medium exceeded its pair on high ascorbate concentration.

b) Histology.

After 1 day the cartilage of the rudiments grown on medium with a high ascorbate level showed much more cell division and broadening in the proliferative and maturation zones. There was also less resorption of the hypertrophic cells when compared with controls on plain medium. In the later stages of culture the central epiphyseal chondrocytes showed increasing vacuolation and hydration with widespread loss of matrix metachromasia with the higher concentrations.

In the bone the poor osteoblast and osteocyte survival previously described with plain TC-199 was still evident and seemed unaffected by the rise in ascorbate level. The bone in the rudiments on high ascorbate concentration medium was non-viable after the second day in culture, presumably from the toxicity of the high medium pH.

5. Effect of Age of embryonic rudiment.

Three litters of foetal mice, classified as 'early', 'intermediate' or 'late' by the criteria of rudiment length, were used for this study. The six tibial rudiments were cultured under identical conditions for six days using BGJ medium supplemented with 5% serum. The results obtained were:-

a) Increase in length.

The increase in length of individual rudiments over the

period of culture compared to their initial length is shown in Table 4 and the mean percentage increase is plotted graphically in Figure 39.

The rate of elongation was much greater in the early rudiments, being twice that in the late group. At all ages maximal growth had been achieved by four days and normally some shortening had occurred by 6 days.

b) Preservation of Histological Appearances.

Histological evidence of viability was preserved in all rudiments at the end of the 6 days in vitro, although the late rudiments showed greater degeneration in the bony shaft. As no marrow cavity had formed in the early rudiments degeneration of the cellular population was not seen and the thin shell of bone was preserved with some thickening by periosteal apposition. In rudiments of all ages the end cartilages and their cellular zones were well preserved and showed elongation by cellular proliferation and formation of ground substance. With the arrest of endochondral bone formation in the intermediate and late rudiments, the increase in length resulted mainly from cartilage hypertrophy.

TABLE 4

Effect of Age of Rudiment on Elongation in Culture.

Rudiments	Time	% increases in length	Mean	S.D.
EARLY	2 day	19.2, 22.4, 32.6, 31.4, 22.4,39.2	27.9	± 7.7
	4 day	36.5, 30.6, 46.9, 39.2, 43.1,22.4	36.4	± 8.8
	6 day	34.6, 26.5, 46.9, 41.2, 24.5,43.1	36.1	± 9.1
INTERMEDIATE	2 day	8.8, 6.6, 10.5, 17.4, 15.6,13.9	12.1	± 4.2
	4 day	22.5, 10.5, 12.1, 19.0, 17.2,18.1	16.6	± 4.5
	6 day	22.0, 10.5, 12.1, 17.5, 15.6,15.3	15.5	± 4.1
LATE	2 day	9.5, 8.0, 12.9, 6.5, 8.1, 9.6	9.1	± 2.2
	4 day	20.0, 17.6, 9.8, 9.3, 8.0,17.6	13.7	± 5.2
	6 day	8.1, 9.3, 8.7, 7.0, 8.3,10.5	8.6	± 1.2

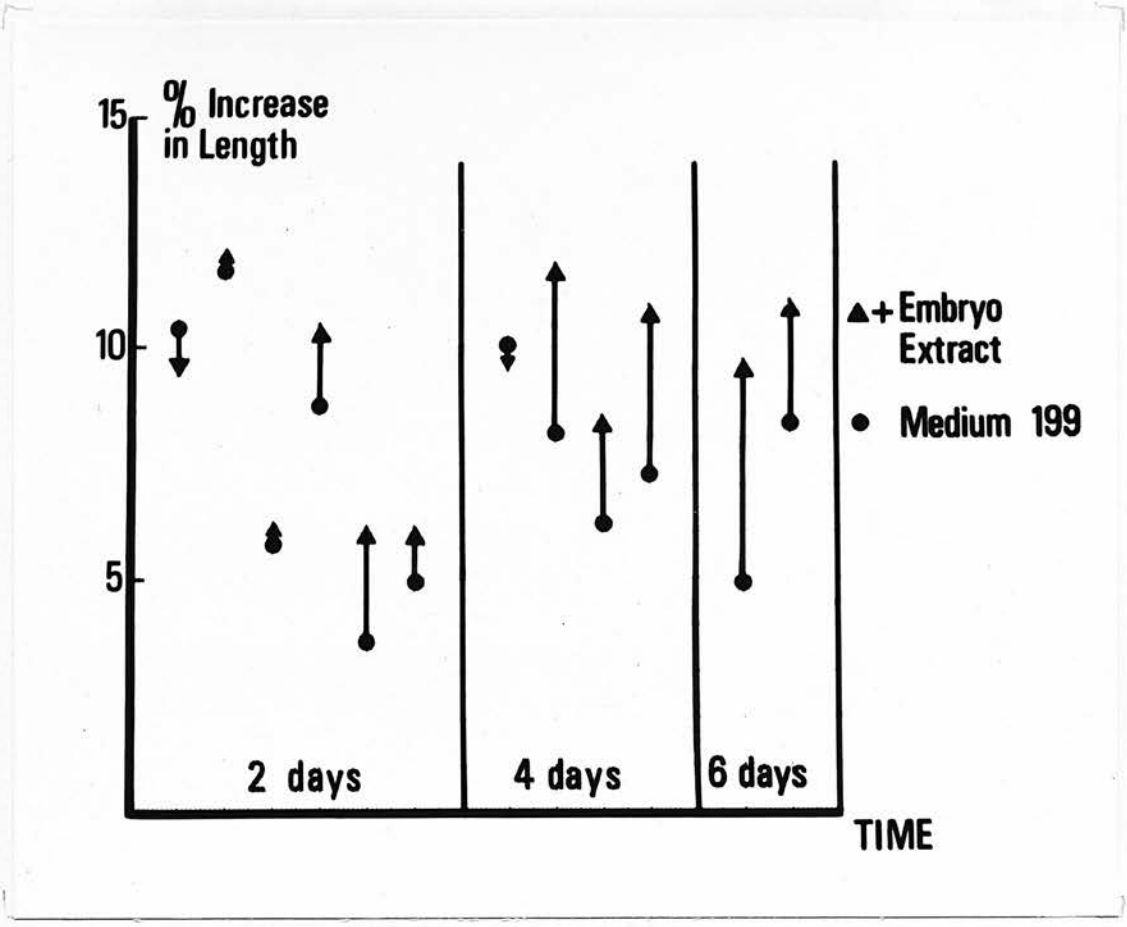
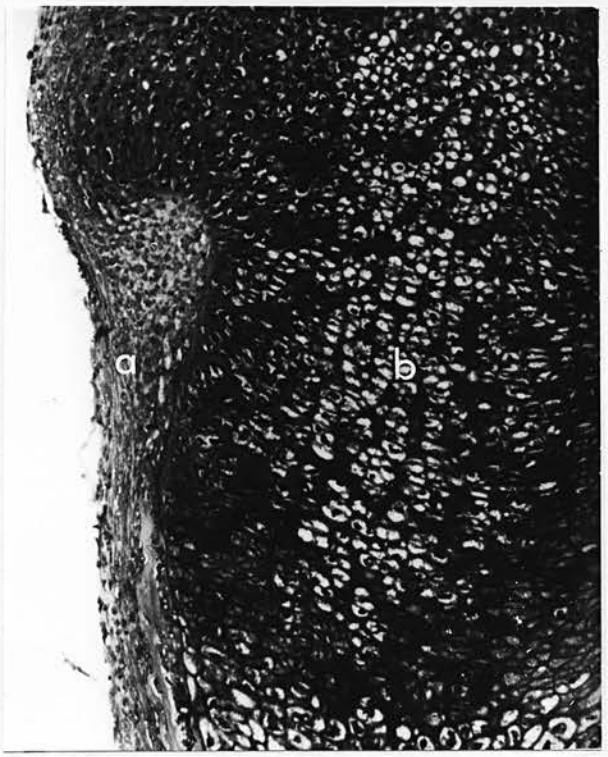


Fig. 20. Percentage increase in length of paired late tibial rudiments on medium TC-199 ± embryo extract.

TABLE 1

PERCENTAGE INCREASES IN LENGTH OF TIBIAL RUDIMENTS

	2 days					
TC-199	10.4	11.7	5.8	8.7	3.6	4.9
TC-199 + Embryo Extract	9.5	11.8	6.0	10.4	6.0	6.0
	4 days				6 days	
TC-199	10.0	8.1	6.1	7.2	4.9	8.4
TC-199 + Embryo Extract	9.6	11.7	8.4	10.8	9.6	10.8



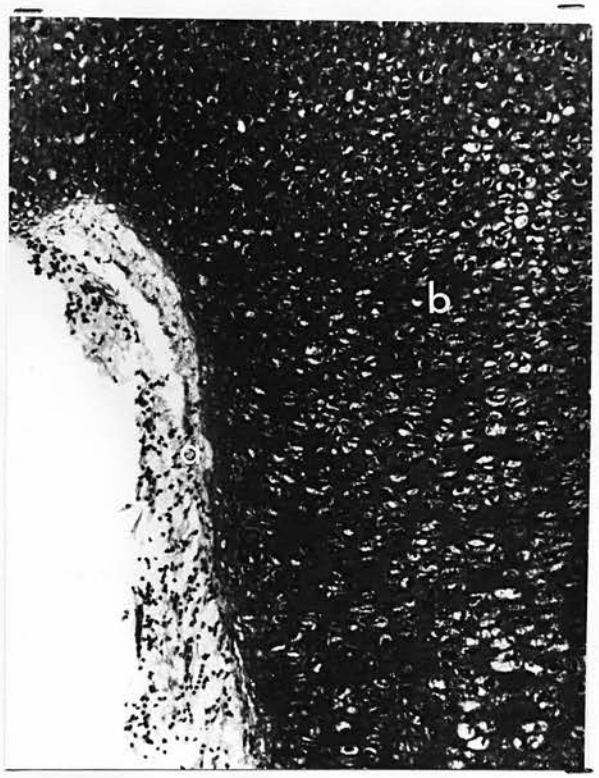
a) Perichondrium thickened

b) Central cell death with loss of metachromasia.

Toluidine Blue

x 90

Fig. 21A. End cartilage after 2 days on TC-199 + Embryo Extract.



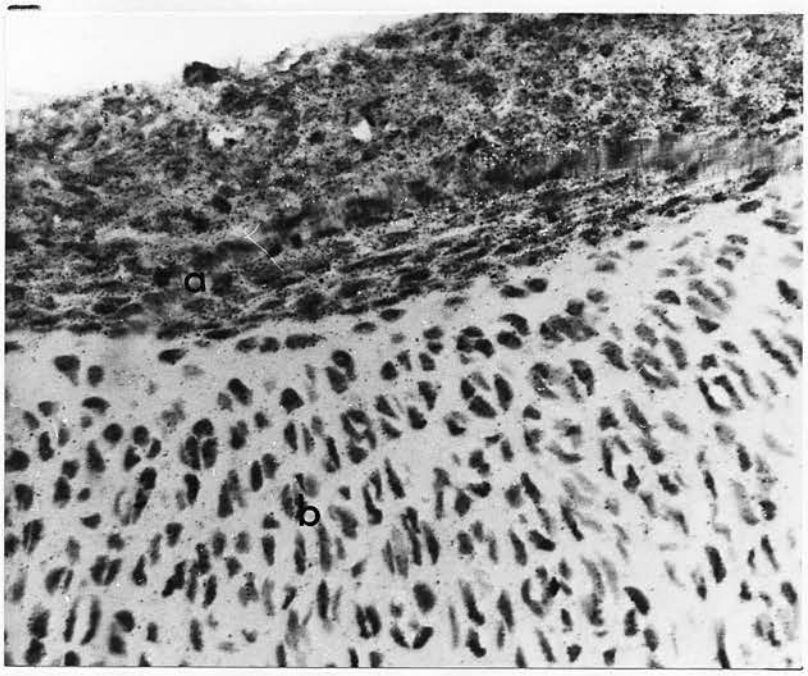
a) Perichondrium thin with some cell death

b) Matrix metachromasia preserved.

Toluidine Blue

x 90

Fig. 21B. End cartilage after 2 days on TC-199 medium.



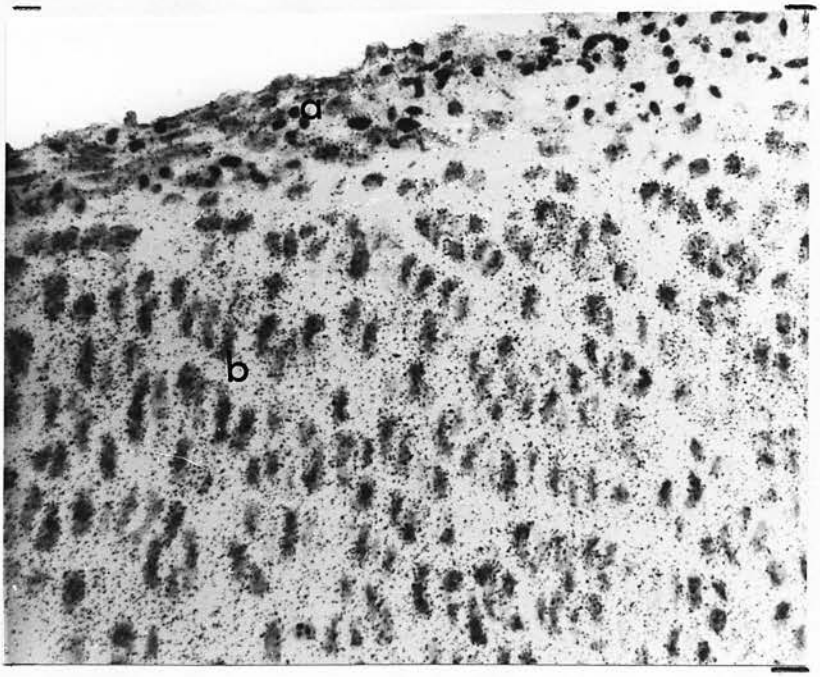
a) Perichondrium

b) Proliferative cell zone

Haematoxylin

x 210

Fig 22A. Autoradiograph of end cartilage after 2 days on TC-199 + Embryo Extract.



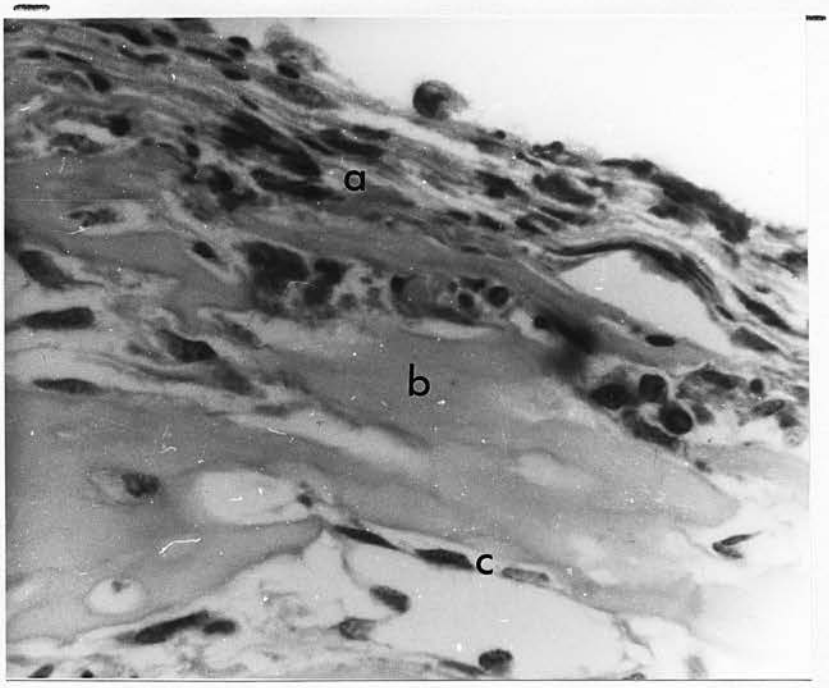
a) Perichondrium

b) Proliferative cell zone

Haematoxylin

x 210

Fig. 22B. Autoradiograph of end cartilage after 2 days on plain TC-199 medium.



a) Periosteum

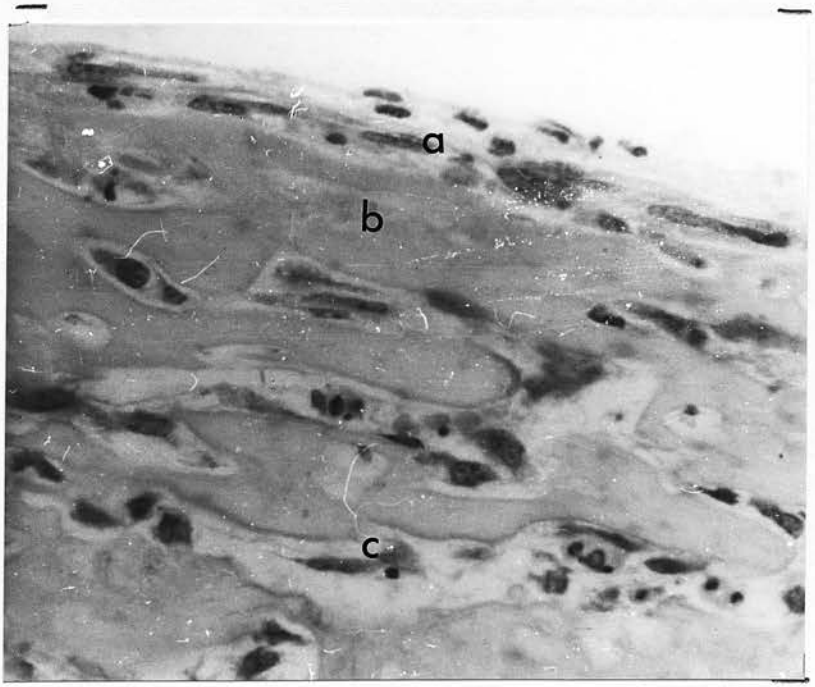
b) Bone matrix

c) Endosteum

H. & E.

x 375

Fig. 23A. Shaft bone after 2 days on TC-199 + Embryo extract



a) Periosteum

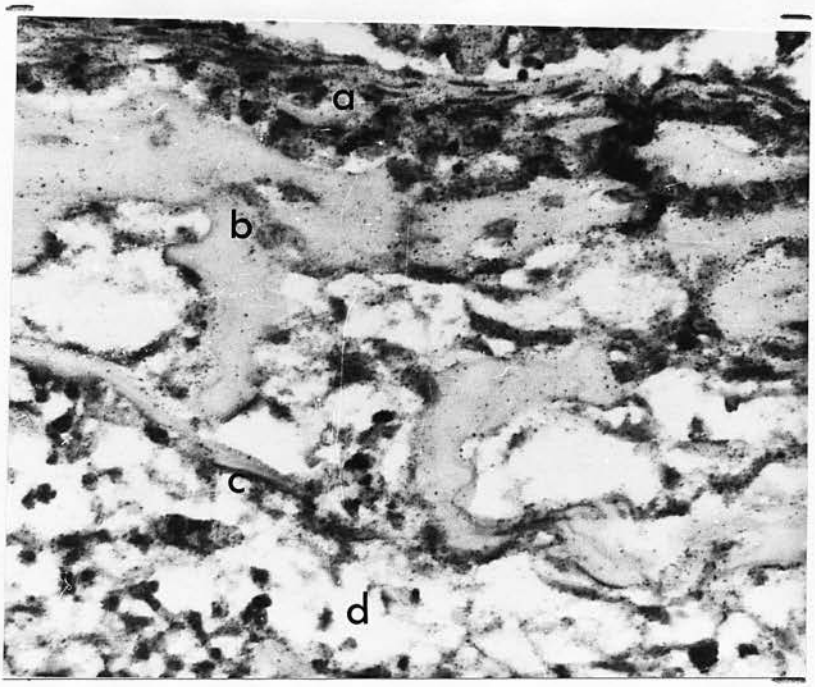
b) Bone matrix

c) Endosteum

H. & E.

x 375

Fig. 23B. Shaft bone after 2 days on plain TC-199 medium.



a) Periosteum

b) Labelled matrix

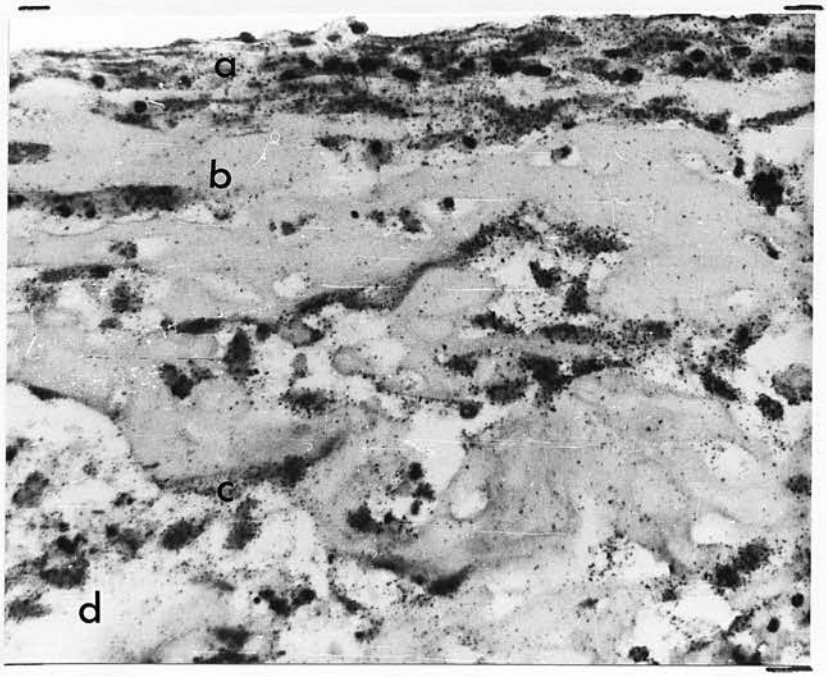
c) Endosteum

d) Marrow cavity

Haematoxylin

x 375

Fig. 24A. Autoradiograph of shaft bone after 2 days on TC-199 + Embryo extract.



a) Periosteum

b) Labelled matrix

c) Endosteum

d) Marrow cavity

Haematoxylin

x 375

Fig. 24B. Autoradiograph of shaft bone after 2 days on plain TC-199

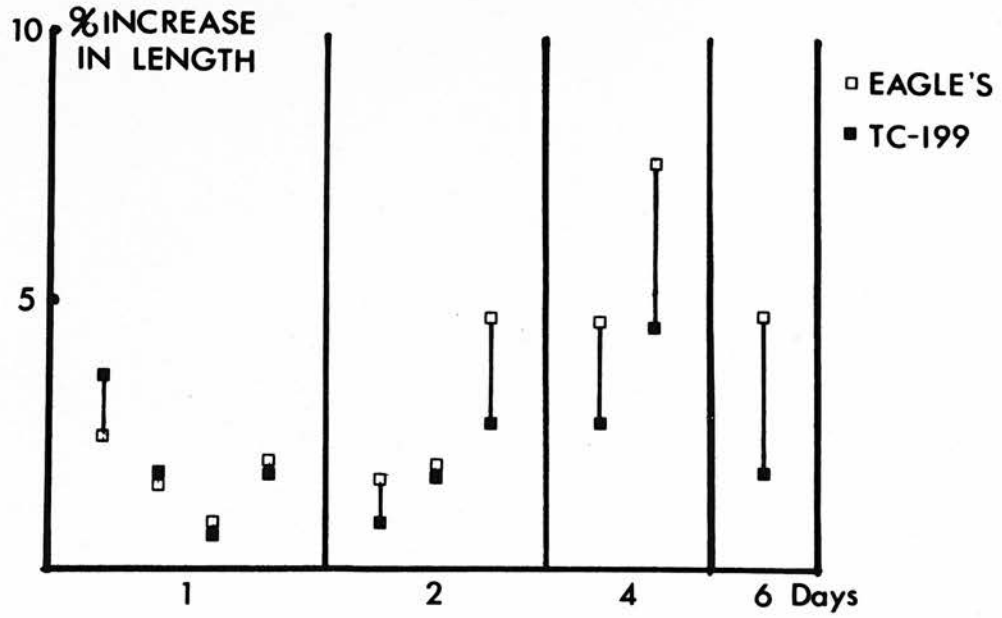


Fig. 25. COMPARISON OF TIBIAL GROWTH IN LENGTH ON EAGLE'S AND TC-199 MEDIA.

Time	Medium	% increase in length			
1 day	Eagle's	2.5	1.7	0.9	1.9
	TC - 199	3.6	1.8	0.8	1.8
2 days	Eagle's		1.7	1.9	4.8
	TC - 199		0.9	1.8	2.8
4 days	Eagle's			4.6	7.6
	TC - 199			2.8	4.5
6 days	Eagle's				4.7
	TC - 199				1.8

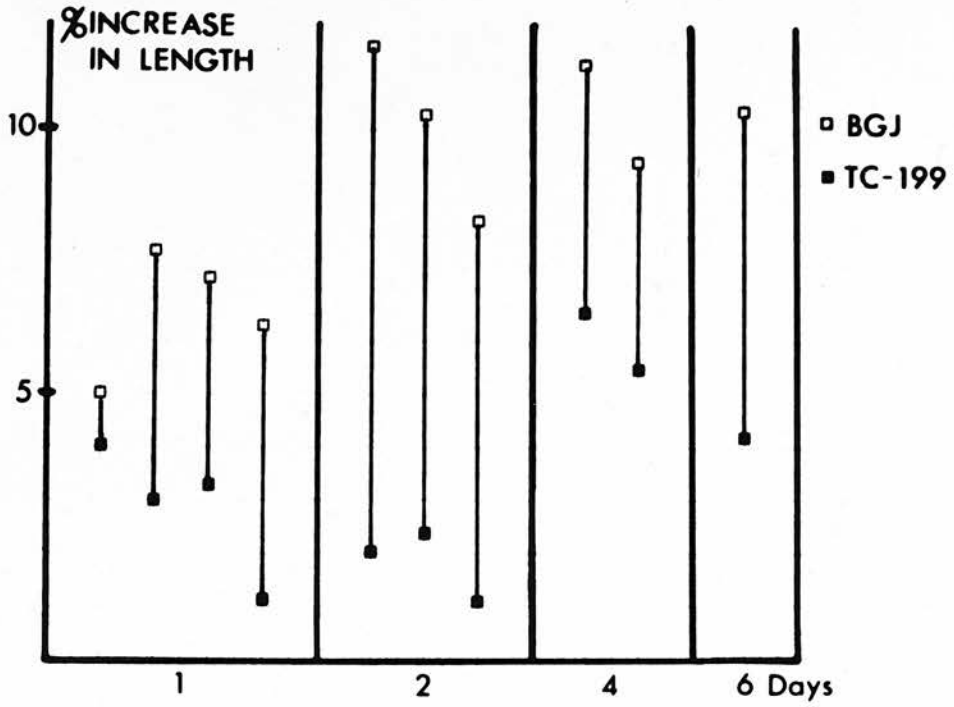
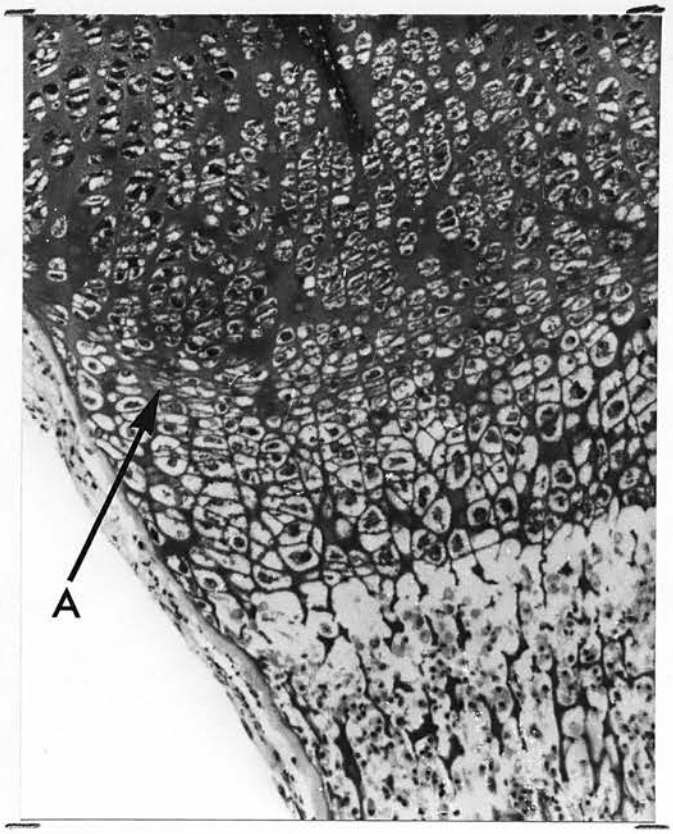


Fig. 26. COMPARISON OF TIBIAL GROWTH IN LENGTH ON BGJ AND TC-199 MEDIA.

Time	Medium	% increase in length			
1 day	BGJ	5.0	7.8	7.2	6.3
	TC - 199	4.0	3.0	3.3	1.1
2 days	BGJ		11.6	10.3	8.3
	TC- 199		2.0	2.4	1.1
4 days	BGJ			11.3	9.4
	TC - 199			6.6	5.5
6 days	BGJ				10.4
	TC - 199				4.2

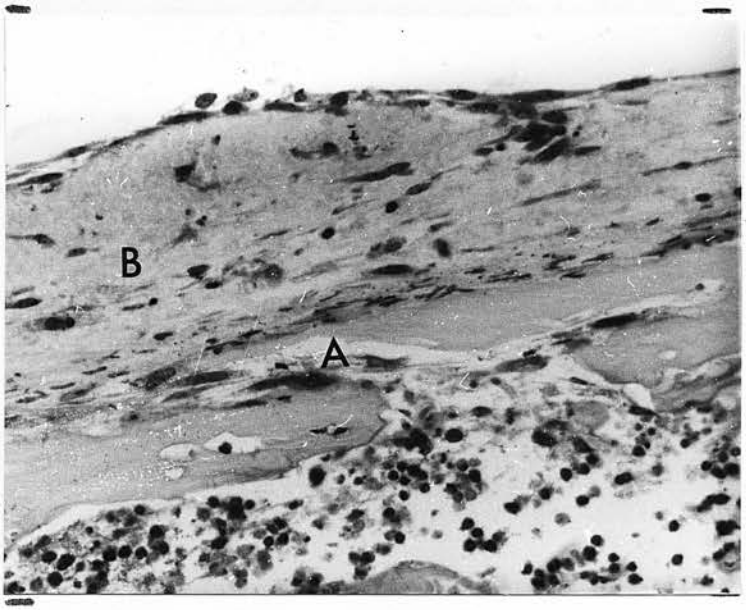


A. Site of junctional zone with cell flattening and loss of MPS stain.

x90

Alcian blue & PAS

Fig. 27. End cartilage after 4 days on Eagle's medium.



A. Osteoblasts

B. Osteoid matrix

x 220

Alcian blue & PAS

Fig. 28. Bony shaft after 4 days on Eagle's medium.

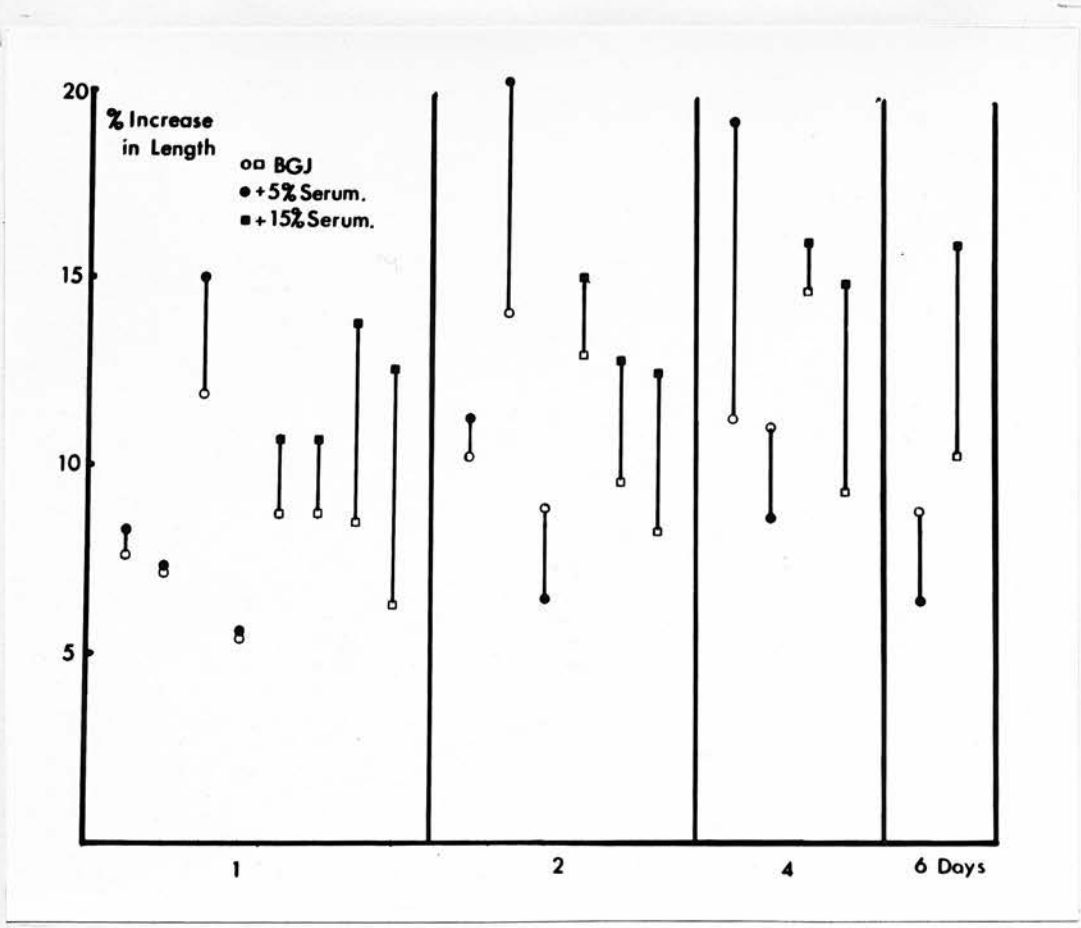


FIG. 29. EFFECT OF SERUM SUPPLEMENTS WITH BGJ MEDIUM

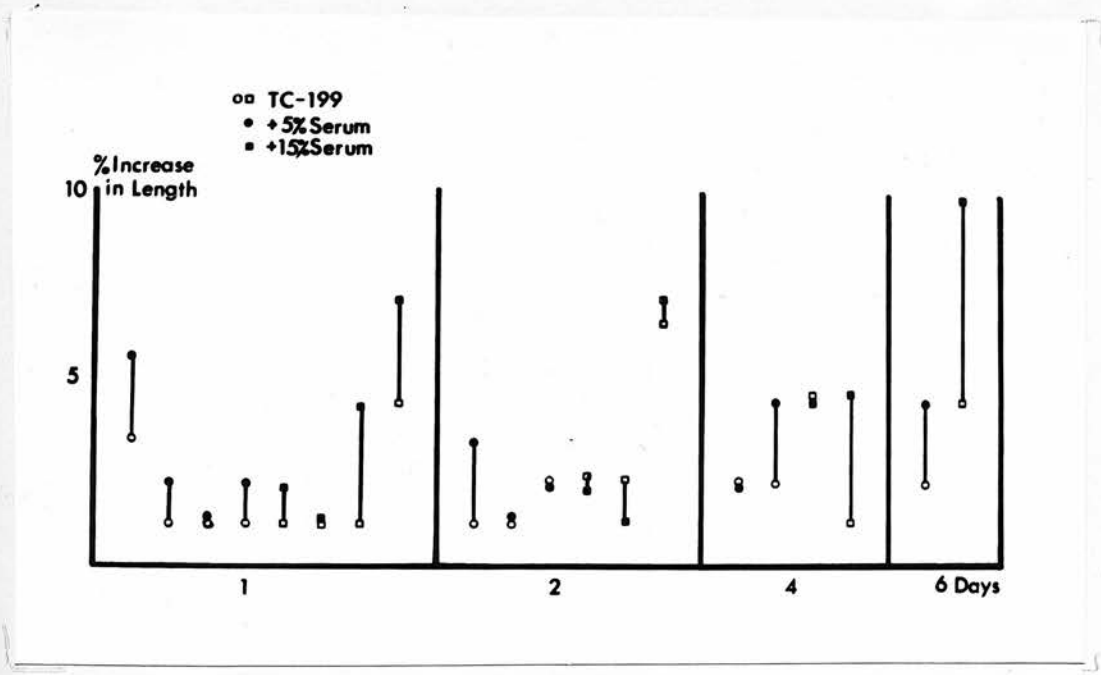


FIG. 30. EFFECT OF SERUM SUPPLEMENTS WITH TC-199 MEDIUM

A. Invasion of hypertrophic zone with PAS-positive staining.

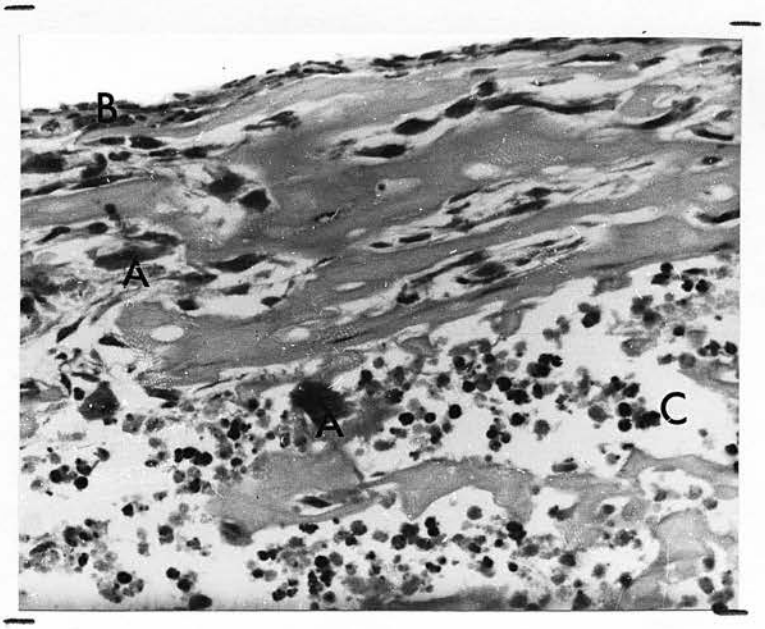
B. Perichondrium

C. Marrow cavity

x 90

Alcian blue & PAS.

Fig. 31. End cartilage after 6 days on BGJ + 15% Serum.



A. Osteoclasts

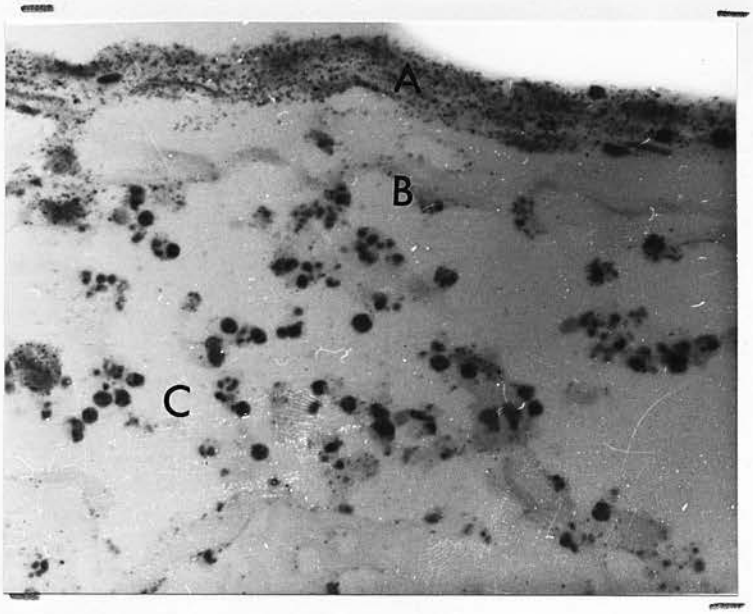
B. Periosteum

C. Marrow cavity

x 340

Alcian blue & PAS

Fig. 32. Shaft bone after 1 day on BGJ + 15% Serum.



A. Periosteal label

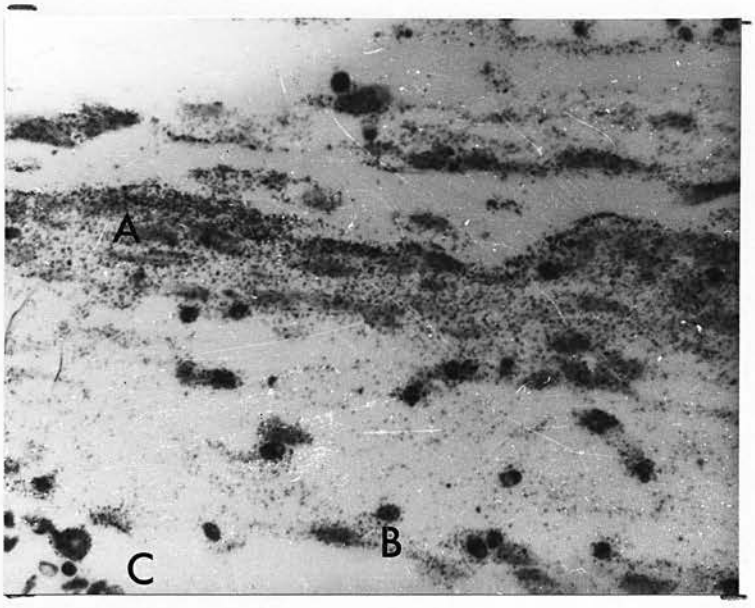
B. Endosteum

C. Marrow cavity

x 340

Haematoxylin

Fig. 33A. Autoradiograph of shaft bone after 2 days on plain BGJ medium.



A. Periostel label

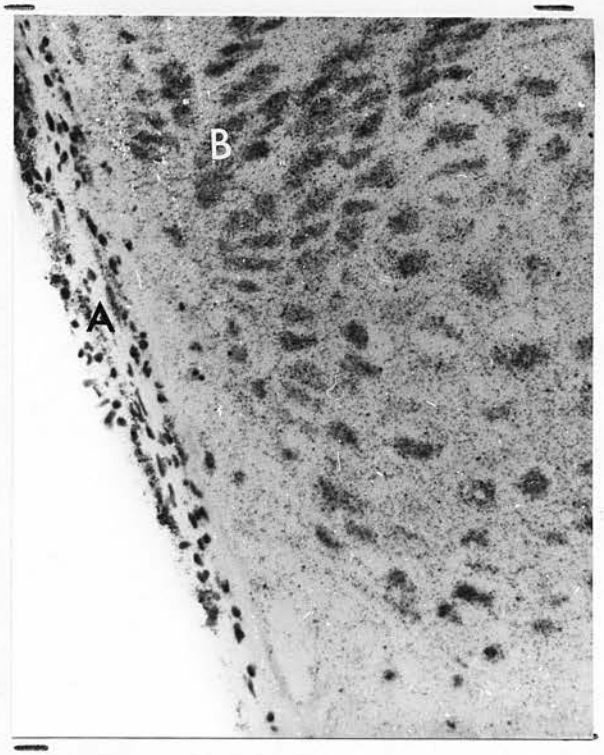
B. Endosteum

C. Marrow cavity

x 340

Haematoxylin

Fig. 33B. Autoradiograph of shaft bone after 2 days on BGJ medium + 15% serum.



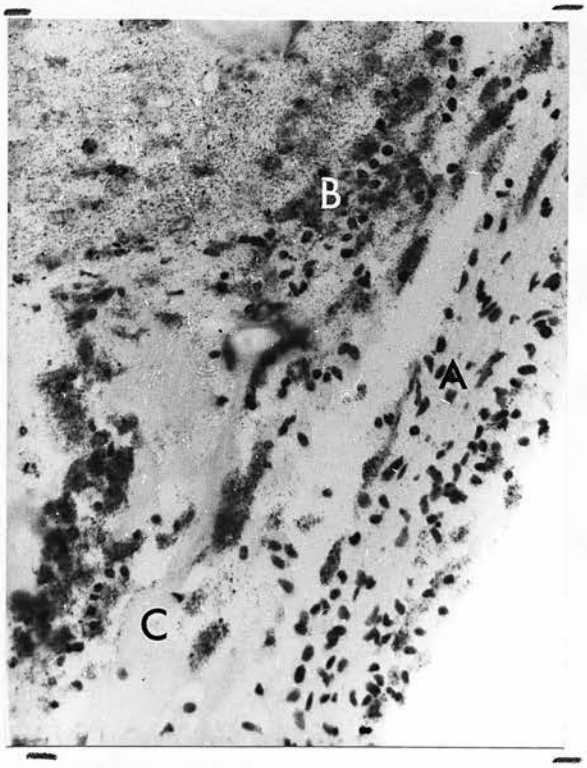
A. Perichondrium

B. Proliferative chondrocytes

x 220

Haematoxylin

Fig. 34. Autoradiograph of end cartilage after 4 days on plain BGJ medium.



A. Perichondrium

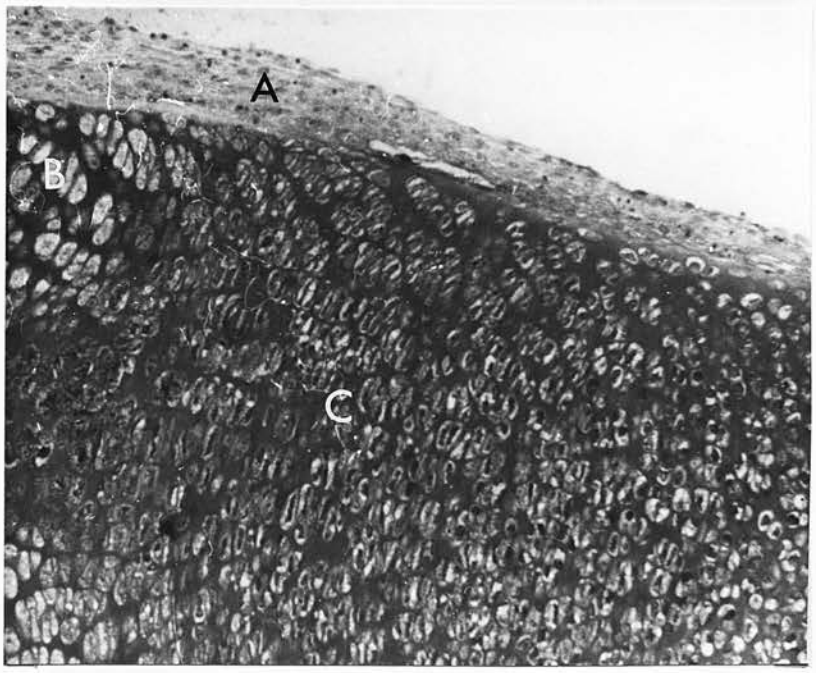
B. Site of perichondral ingrowth with label deposition

C. Shaft bone

x 220

Haematoxylin

Fig. 35. Autoradiograph of end cartilage after 4 days on BGJ + 5% serum.



A. Perichondrium

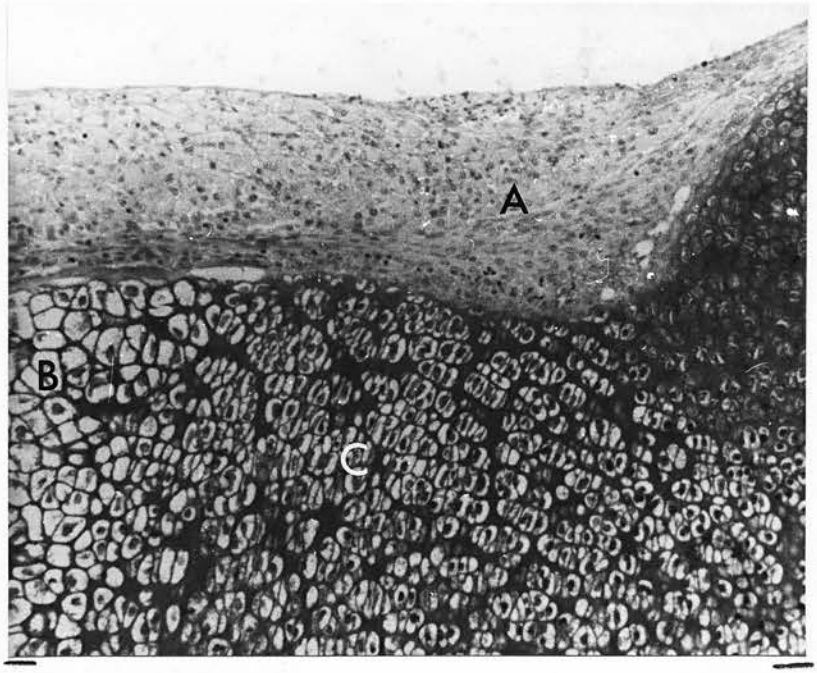
B. Hypertrophic cells

C. Chondrocytes

Alcian blue & PAS

x 90

Fig. 36A. End cartilage after 2 days on plain TC-199 medium.



A. Perichondrium (thickened)

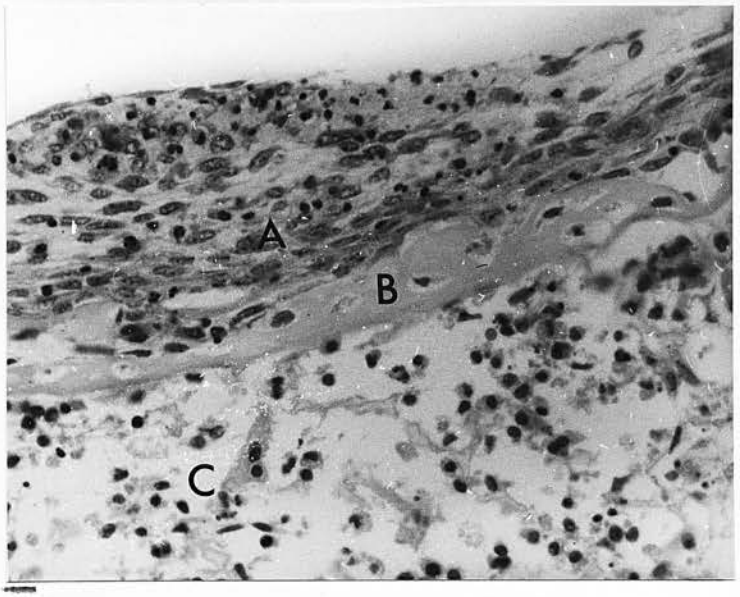
B. Hypertrophic cells

C. Chondrocytes (more vacuolation)

Alcian blue & PAS

x 90

Fig. 36B. End cartilage after 2 days on TC-199 + 15% Serum.



A. Thickened periosteal osteoblast layer

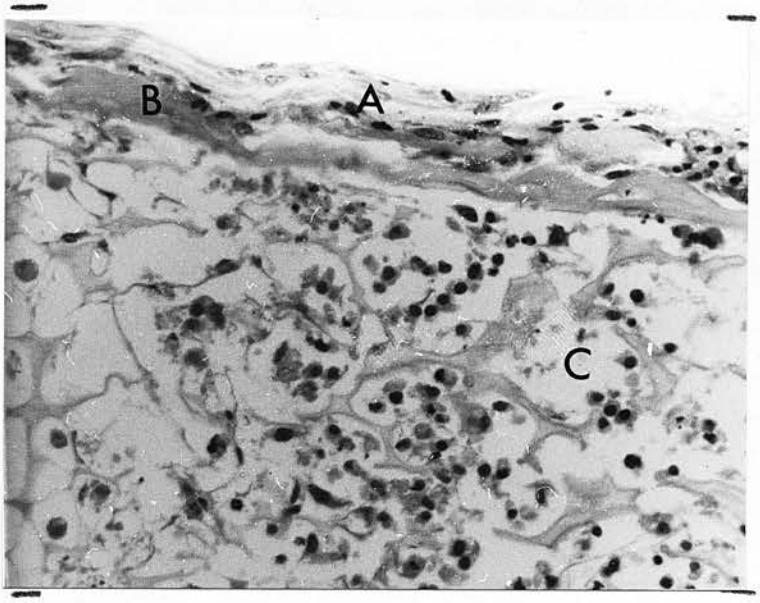
B. Bone

C. Marrow cavity

Alcian blue & PAS

x 340

Fig. 37. Shaft bone after 2 days on TC-199 + 15% Serum.



A. Thinned periosteal osteoblast layer

B. Bone

C. Marrow cavity

Alcian blue & PAS

x 340

Fig. 38. Shaft bone after 6 days on TC-199 + 15% Serum.

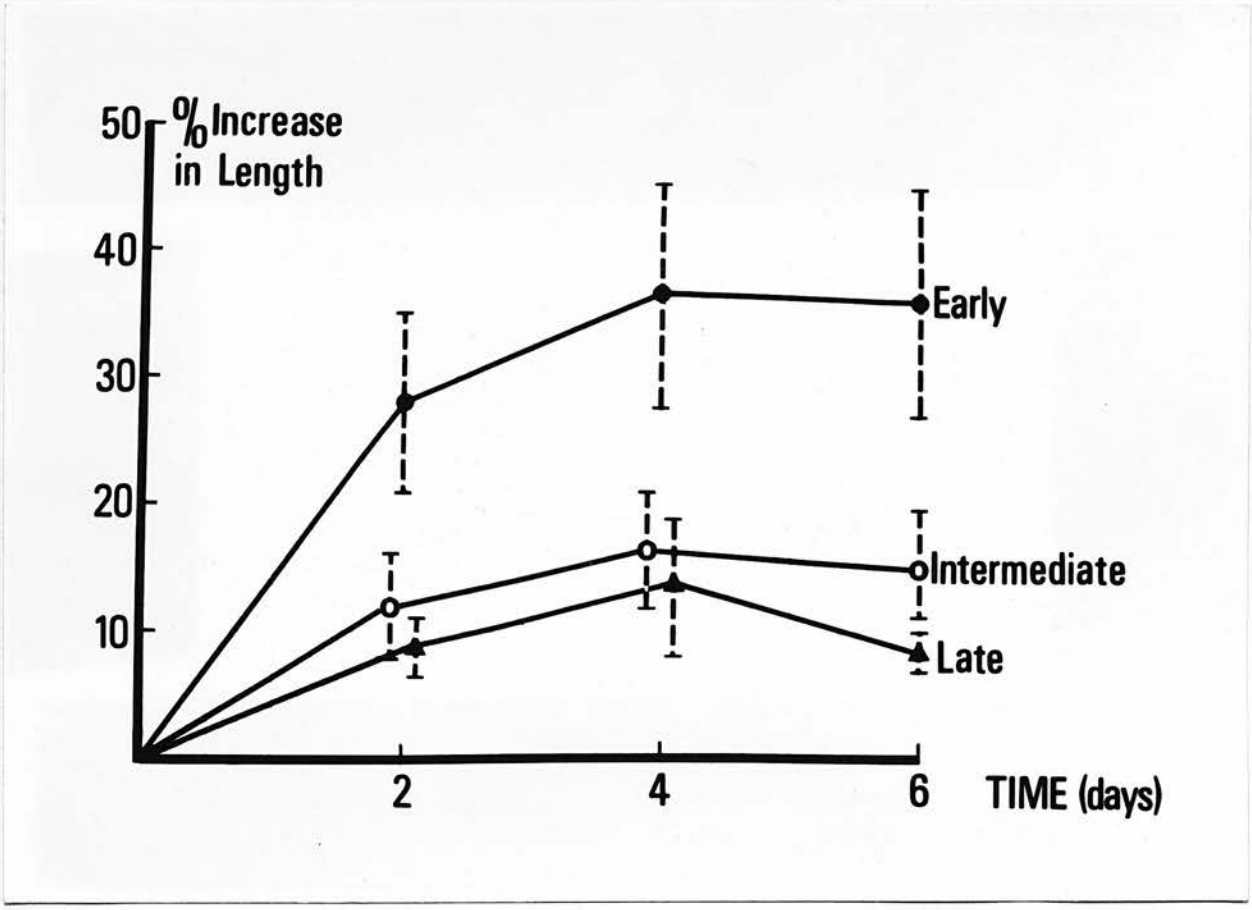


Fig. 39. Comparison of percentage increase in length with age of rudiments.

DISCUSSION

1. Synthetic or Natural Media?

The use of embryo extract as a 20% supplement for the synthetic medium improved growth in length but did not maintain normal rudiment morphology. There was improved periosteal bone growth when compared with synthetic medium alone but this was accompanied by more degeneration of cartilage and an arrest of endochondral bone formation.

Although the addition of 20% embryo extract to synthetic TC-199 does not represent a full natural medium, it does introduce the unknown stimulatory effect of embryonic juices. The limitation of growth in length at 10% is similar to that obtained by Fell and Mellanby (1952) on a mixture of plasma and embryo extract. The improved elongation with embryo extract obtained in these experiments is at variance with the findings of Jones and Keeler (1971), although they had used synthetic Eagle's medium rather than TC-199. In addition they added 50% serum to their synthetic medium and used much younger 15-day foetal rudiments, which consist largely of cartilage. Nevertheless the histological changes they reported were similar, showing preservation of cartilage but arrest of periosteal and perichondrial bone formation. From these experiments it seems that the disadvantages of introducing the unknown biological factor of embryo extract are not outweighed by the improved growth and preservation of normal morphology, though obviously synthetic medium alone is insufficient for normal growth.

2. Which synthetic medium?

Of the three synthetic media compared over a 6 day period in culture, the P6 modification of BGJ medium gave the most satisfactory elongation, while TC-199 was poorest. However the differences were not great and the morphological changes were probably of greater significance. None of the media tested was sufficient to maintain full viability in all the tissues of the rudiments for more than 24 hours.

The end cartilages showed early degenerative changes after only 1 day in culture, but these were less marked with BGJ medium. This would support the view of Reynolds (1966) that the higher level of vitamin C in this medium is necessary for the preservation of chondrocyte viability and the prevention of over-hydration. The appearance of the layer of flattened cells sealing the hypertrophic zone from the shaft after 2 - 4 days in culture was difficult to explain. It has been reported previously by Jones and Keeler (1971), who attributed it to high vitamin C levels in the medium. However, as it was seen with all the media tested this cannot be the cause since the ascorbate level varied widely. It may represent the formation of a layer of osteoid by osteoblastic cells on the surface of the distal degenerating hypertrophic cells which is not resorbed in the absence of the metaphyseal blood vessels. Although the end cartilages were best maintained on BGJ medium, they did show evidence of central chondrocyte vacuolation with the appearance of positive glycogen staining. This may represent the early changes preceding the appearance of a secondary ossification centre.

The bony shaft showed degenerative changes from the second day onwards superimposed on a loss of the normal marrow cell population. There was little difference in the periosteal osteoblast activity between media, but resorption of bone was greater with TC-199 than either Eagles' or BGJ medium.

3. Are Serum Supplements required?

The experimental evidence obtained suggests that serum supplements are necessary to maintain a degree of viability in the explants over a 6 day period of in vitro culture. Its addition produced improved elongation of the rudiments and with BGJ medium, which also contains an optimum concentration of ascorbic acid, this reached a maximum of 15 - 20%. This effect and the maintenance of the cell populations within the rudiments appeared to be slightly dose dependent, although this was related to the timing of the effect rather than its maintenance.

At the cellular level the most dramatic effects of serum supplementation were seen in the bone. The increased cellular activity previously reported by Fell and Weiss (1965) was confirmed, as was the increased osteoclast production referred to by Liskova and Jean (1970). Synthetic activity in the osteoblasts and chondroblasts of the periosteum and perichondrium was also enhanced and it seems certain that serum is essential if this is to occur on synthetic medium. Its effect on cartilage was less clear-cut but no adverse effects were noted.

4. Is Ascorbic Acid necessary for cartilage viability?

From the review of the literature on the role of ascorbic acid or vitamin C clear evidence emerged that it was necessary for the prevention of over-hydration of cartilage in culture. A reversed effect was seen in the experiments described but this could be attributed to the gross deviation from normal of the pH in the culture medium. When the increased concentrations of ascorbic acid were added to TC-199 medium toxic effects quickly occurred in the bone and were followed thereafter by similar changes in the cartilage. The reverse of the desired effect of prevention of cartilage hydration and loss of ground substance was seen. Despite this the BGJ medium which already contains supplemented levels of ascorbic acid to 150 μgm/ml. gave satisfactory maintenance of viability with the addition of serum supplements, suggesting that the substance is not toxic in itself. Ascorbic acid which is a reducing agent would certainly be required in later experiments when it was planned to use high oxygen concentrations in the gas phase.

5. What age of rudiment should be used?

Although the early rudiments demonstrated the most satisfactory growth in length, in culture they were morphologically incomplete in lacking endochondral bone formation and a marrow cavity. Being largely cartilaginous the hypertrophy of the end cartilages produced maximal elongation.

The late rudiments, in which the bony shaft occupies more than two-thirds of the total length, elongated poorly due to the cessation of endochondral bone formation.

The intermediate length rudiment was the most satisfactory model, as the relatively large end cartilages still offered an adequate growth in length, while the bony shaft was sufficiently formed to allow evaluation of the effects of other variables on its morphology.

In summary these experiments on the variables of this in vitro culture system would suggest that the nearest approach to the ideal would be to use intermediate age rudiments grown on BGJ medium supplemented with 15% serum and an ascorbic acid level of 150 μgms/ml.

PART II

COMPARISON OF IN VITRO GROWTH WITH NORMAL IN VIVO DEVELOPMENT OF MOUSE LIMB BONES

INTRODUCTION

In Part I of this thesis the effect of variables on the in vitro growth of foetal mouse limb bones was considered and the optimum conditions for growth in culture determined. This growth and development during a six day period in culture should ideally approach to the normal in vivo situation. Thus it was felt necessary to compare the in vitro growth with normal in vivo development up to and beyond the time of explantation. Any changes induced in the mouse limb bones in culture by alteration of the oxygen level in the gaseous phase would need to be interpreted in relation to this comparison.

In vivo development of the mouse limb bones.

The prenatal development of the mouse appendicular skeleton has been well documented in the papers of Carter (1954) and Forsthoefel (1959). They described the normal process of development in the CBA, CK and C57 mouse strains respectively as a preliminary to a study of genetic defects induced in the limbs. Both these workers counted the gestation period in half days as fertilisation was allowed to occur overnight and the experimental dissections were carried out on successive mornings.

Upper limb development preceded that of the lower limb by approximately one day. In the 9½ day embryo an ectodermal thickening had appeared over five segments at the site of the upper limb bud development. At 10½ days the mesenchymal bud had developed as a projection covered by ectoderm. At 11½ days foot plate development had commenced and the first evidence of blastemal condensations for the post-axial bones of the scapula,

humerus and ulna had appeared. At 12½ days the foot plate was rounder and the pre-axial condensations had appeared for the radius and carpus. At the same time chondrification had commenced in the humerus, ulna and scapula. At 13½ days the foot plate was polygonal and indented and blastemal condensations were present for the metacarpals. Chondrification had commenced in the radius and all the cartilage of the long bone shafts showed hypertrophy prior to ossification. At 14½ days the fingers were separated distally and all carpal elements were chondrified. The middle of the perichondrium in the shaft of the long bones was invaded by blood vessels and converted to periosteum. At 15½ days ossification had commenced in the shaft of the humerus, radius, ulna and scapula, while the shoulder and elbow joints were formed and all the limbs moved on stimulation. At 16½ days all the upper limb skeleton was chondrified and ossification had also occurred in the second to fifth metacarpals. At 17½ days immediately preceding birth all the limb bone rudiments showed ossification in their shafts, except for the carpus which still consisted entirely of cartilage.

As already stated, development of the lower limbs did not commence until 10½ days when it was represented by an ectodermal swelling over four segments. At 11½ days the mesenchymal lower limb bud projected ventrally and early blastemal condensations were evident for the pelvis, femur and proximal fibula. At 12½ days the circular foot plate was well differentiated and further condensations of mesenchyme were evident for fibula, tibia and tarsus. At the 13½ day stage there were shallow indentations in

the polygonal foot plate together with chondrification, which commenced first in the femur and followed quickly in the tibia and fibula. At 14½ days the foot plate was deeply indented and hypertrophy of the cartilage had commenced in the shafts of the femur, tibia and fibula. At 15½ days the toes had separated and diverged, the hip joint had commenced forming and ossification was visible in the shafts of the femur, tibia and fibula with early differentiation of the knee joint. At 16½ days advanced ossification was present in the femur, tibia and fibula. At 17½ days immediately preceding birth ossified shafts were present in the ilium, pubis, ischium, femur, tibia, fibula, the intermediate portions of the tarsus, all the metatarsals and the phalanges. The patella and all other tarsal elements remained in cartilage.

Comparisons of in vitro and in vivo growth.

Several workers have compared the in vitro and in vivo growth of limb bone rudiments from the chick embryo. They have all used rudiments from embryos less than 10 days old, which consist entirely of cartilage. Fell & Mellanby (1955) investigated the relative growth in length of rudiments from the leg and wing buds of 4 to 7 day old embryos. They compared rudiments removed from normally developing embryos with those grown on a natural medium of plasma and embryo extract. Both in vivo and in vitro the greatest increase in length occurred in the tibia, followed by the femur, humerus, ulna and radius. On average the rudiments in vivo elongated at two or three times the rate in vitro.

Biggers (1960) compared the increase in length, wet weight and dry weight of the tibiotarsus from 7 day old embryos over a

period of four days. In the intact animal the wet weight increased ten times and the dry weight twelve times, as compared with a three times increase of wet weight and a doubling of dry weight in culture. These results were obtained using his own synthetic medium, but the use of natural media gave little improvement on these figures.

A more elaborate quantitative comparison of the in vivo and in vitro growth of the chick tibiotarsus was reported by Schryver (1966). He used rudiments from 8 day embryos and studied the increase in length, wet weight, dry weight, DNA content and chondroitin sulphate content during two days in vivo development with up to 8 days in tissue culture. The dry weight to wet weight ratio in vivo remained virtually unchanged, while the ratio in vitro declined continually during the culture period. This was due to the hydration of rudiments grown in culture which probably represents a degenerative phenomenon. The ratio of dry weight to DNA showed that the cells in culture did not synthesise as rapidly as in vivo. On the other hand, the ratio of chondroitin sulphate to DNA indicated that the cells in culture were producing chondroitin sulphate at a rate similar to that in vivo. In fact the proportion of chondroitin sulphate in culture was greater than in the rudiments from the intact embryo. This would suggest that the matrix of skeletal rudiments grown in vitro, although chondroitin sulphate rich, may be deficient in one or more matrix components. All the variables investigated during the period of in vitro growth fell far short of the linear response obtained in vivo.

None of these reported studies had used chick limb bone rudiments from embryos older than ten days, when the formation of a marrow cavity and endochondral ossification becomes established in the bony shaft. They provide little information as to the changes which occur in rudiments with a significant bone content in addition to cartilage. The only study comparing growth of mammalian rudiments in vivo and in vitro is that of Mitchell (1950). He reported the growth in length of femora from 17 day old rat embryos, which show perichondral but not endochondral bone formation, over a three day period. In utero these increased by nearly 150% as compared with 25% in culture, but this probably represents the failure to commence endochondral bone formation in vitro.

The post-natal growth of the mouse limb bone has not been described in the literature. A study was therefore carried out to compare the in vivo growth of limb bones from newborn mice over a six day period with in vitro growth of rudiments from the same litter. Because of the difficulty in accurate ageing and the variations in rudiments produced by litter size, a comparison of pre-natal growth in vivo against the same age in vitro was not attempted.

The experiments reported in Part II of this thesis compare the in vitro growth with the in vivo development of post-natal mouse limb bones during a six day period. Comparisons were made on the basis of:-

(1) Growth in length.

(2) Increase in wet weight and dry weight.

(3) Histological structure.

MATERIALS AND METHODS

1. Mice.

The T.O. Tuck's No.1 strain of mouse used in Part I of this work was also used in these experiments. Pregnant females were allowed to give birth to their litter normally and the newborn mice used to compare the in vivo and in vitro growth of fore and hind limb bones.

Four mice were killed immediately with ether vapour and the limb bones removed for culture or as zero controls. The remainder were allowed to remain with the mother for normal in vivo development, one mouse being sacrificed every 48 hours and the limb bones removed.

2. Isolation of Bone Rudiments.

The post-foetal mice used for in vitro culture were washed three times in 70% alcohol and then in sterile Tyrode's solution. The fore and hind limbs were removed and immersed in a dish of Hanks' balanced salt solution at room temperature.

The limb bones were dissected from the soft tissues under magnification using the sterile technique described in Part I.

A similar dissection technique was used for the dissection of the limb bones from the mice allowed to develop in vivo but no sterile precautions were observed.

3. Culture Technique.

Plastic culture dishes containing stainless steel grids were prepared for the rudiments as described in Part I. A volume of 1.5 ml of medium was used in each dish.

The gaseous phase used throughout these experiments was 95% air with 5% carbon dioxide at atmospheric pressure. Cultures were regassed every 24 hours and the media were changed every 48 hours.

4. Culture Media.

Two sets of experiments were carried out using the P6 modification of BGJ medium, enriched with 15% heat inactivated serum.

5. Measurement of Growth.

(a) Growth in length.

Limb bones grown in culture were measured with an eyepiece graticule at the time of explantation, at each media change, and at the completion of six days in culture. Using the same eyepiece the bones from the mice allowed to develop in vivo were measured after dissection from the soft tissues and prior to fixation.

(b) Weight of rudiments.

The wet and dry weights of the bones were determined for the zero controls at the time of explantation and for the in vitro and in vivo rudiments at the time of harvest after each 48 hours. Wet weights were determined after the bones had been rolled over once on dry filter paper (Whatman's No.1) and placed in pre-weighed glass vials. The dry weight was estimated after drying the bones in a hot air over at 105° C for 24 hours and allowing them to cool in a dessicator. All weighings were carried out using a Shandon Unimatic CL-41 micro-balance.

6. Histological Examination.

(a) Fixation.

As described in Part I.

(b) Decalcification, dehydration, and embedding.

Decalcification was not required for the bones grown in culture, but those which developed in vivo tended to buckle when sectioned unless subjected to this procedure prior to embedding. These bones were placed in 15% E.D.T.A. solution for six hours to achieve adequate decalcification.

All bones were then dehydrated, cleared and embedded as described in Part I.

(c) Sectioning.

Sections were cut longitudinally as described in Part I.

(d) Staining.

Sections were stained with haematoxylin and eosin, toluidine blue, alcian blue and P.A.S., and Heidenhein's azan stain following the schedules described in Appendix A.

(e) Microscopical examination.

After mounting with DPX, the histology was examined by light microscopy. In addition, the relative length of end cartilages and calcified shaft in each bone were measured by the use of an eyepiece graticule. This allowed comparison of the growth of bone and cartilage under the two conditions.

RESULTS

1. Growth in Length.

The mean percentage increase in length for tibia, radius and ulna over a six day period of growth in vivo and in vitro is shown graphically in Figure 40. The bones allowed to develop in vivo show a progressive increase in length of 15 - 20% during each 48 hour growth period. In vitro the maximum increase in length did not exceed 20%, the majority of which occurred in the first two days of culture. Some rudiments showed a slight decrease in length between the fourth and sixth day in culture because of distortion. Both in vivo and in vitro the greatest percentage increase in length occurred in the tibia and was least in the ulna.

The relative increase in length of the end cartilages compared with the bony shafts for each of the three limb bones studied is shown in Table 5. The figures show that in vivo the bony shaft grows at a much faster rate than the end cartilages which remain relatively unchanged in length. In vitro, the bony shaft fails to elongate and a minimal increase in length occurs by slight enlargement of the end cartilages. This discrepancy in growth is well demonstrated by Figures 41 - 43, showing the relative increases in length for each of the three bones over a six day period in vivo and in culture.

2. Increase in Wet Weight and Dry Weight.

The changes in wet weight and dry weight of a pair of tibiae, radii and ulnae taken from the same litter of mice

TABLE 5

RELATIVE LENGTHS OF BONE AND END CARTILAGES IN VIVO AND IN VITRO.

BONE	PROXIMAL CARTILAGE	DISTAL CARTILAGE	BONE	TOTAL
TIBIA				
Zero control	3.5	3.1	7.5	14.1
2 day - in vivo	3.8	3.2	9.2	16.2
- in vitro	3.6	3.6	8.0	15.2
4 day - in vivo	3.9	3.2	12.5	19.6
- in vitro	3.3	3.1	6.5	12.9
6 day - in vivo	4.0	4.3	16.3	24.6
- in vitro	3.5	3.2	6.5	13.2
RADIUS				
Zero control	1.4	2.9	6.5	10.8
2 day - in vivo	1.4	3.7	8.4	13.5
- in vitro	1.2	3.7	7.1	12.0
4 day - in vivo	1.0	3.6	10.7	15.3
- in vitro	1.5	3.3	6.0	10.8
6 day - in vivo	0.8	4.0	13.6	18.4
- in vitro	2.0	3.6	6.3	11.9
ULNA				
Zero control	4.0	3.5	7.5	15.0
2 day - in vivo	2.5	4.2	10.0	14.7
- in vitro	3.7	3.3	7.4	14.4
4 day - in vivo	3.0	4.4	12.2	19.6
- in vitro	3.9	3.8	7.1	14.8
6 day - in vivo	2.1	4.6	16.2	22.9
- in vitro	4.0	3.9	7.0	14.9

N.B. All measurements from fixed histological sections.

were compared in vivo and in vitro over a six day period. The results of both weighings are shown in Table 6 and the dry weights are presented graphically in Figure 44. In all three bones the increases in wet and dry weight when compared with zero controls were at least twice as great in vivo as in vitro. The bones in vitro showed little increase in wet or dry weight during the period of culture. In all three bones the greatest dry weight in vitro was after two days in culture and this fell again at six days to just above the zero controls.

3. Histological Structure.

There were few differences visible in the structure of the end cartilages when comparing bones maintained in vivo and in vitro. One feature seen after six days in vivo was the relative shortening of the cartilage in relation to the overall bone length and was particularly marked in the proximal epiphyses of the radius and ulna. Metachromatic staining was retained in the cartilage, whether in vivo or in culture and was most intense in the zone of maturation. In the hypertrophic cell layer it persisted most markedly in the longitudinal septa, but was resorbed in the transverse septa of the more distal cells. This metachromasia was retained in the cartilaginous cores of the new trabecular bone formed by endochondral ossification in the metaphyses. There were active osteoblasts on the surface of the trabeculae laying down osteoid which stained strongly positive with P.A.S. This endochondral ossification was arrested in culture where some resorption occurred at this site.

After six days in vivo the bony shaft showed less cellular activity in the periosteum overlying its centre, where it consisted largely of flattened fibroblast-like cells. The cortical bone at this site showed little new bone apposition at its surface, or resorption on its endosteal surface. The site of the nutrient artery was well marked in the proximal tibia. Marked formative and resorptive cell activity was still present towards the bone ends, where the periosteum was 10 - 15 cells thick. A more even distribution of periosteal osteoblasts down the length of the shaft was seen in culture.

Although the secondary ossification centres do not appear until the sixth day in the tibia, it was expected that these would be present in the proximal epiphyses of the radius and ulna by the third or fourth day. Sections of the end cartilages after four days in vivo, when stained with alcian blue and P.A.S. showed increased vacuolation of chondrocytes and loss of metachromasia in the proximal part of the epiphyseal cell zone. This was accompanied by an increased deposition of P.A.S.-positive material, probably glycogen, in the cytoplasm (Fig. 45). When the end cartilages of bones maintained in culture were examined, similar changes were observed suggesting that these were probably the early stages of secondary ossification centre formation (Fig. 46).

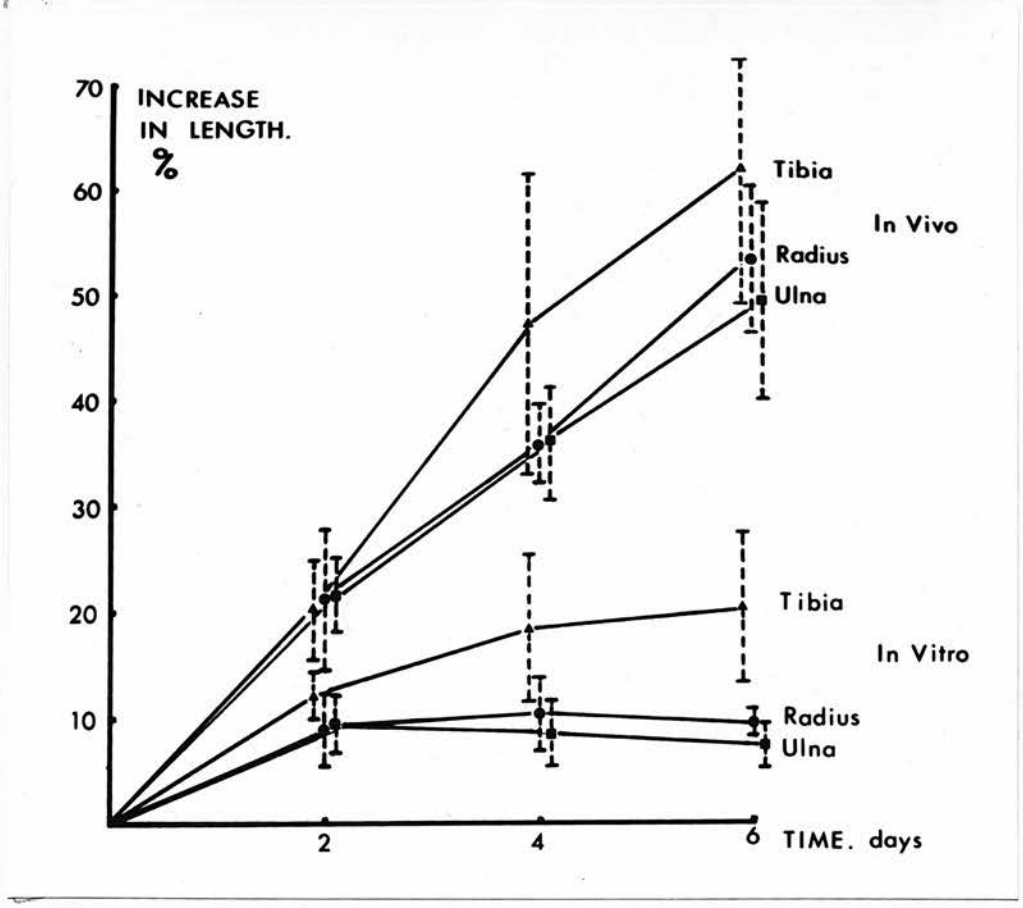
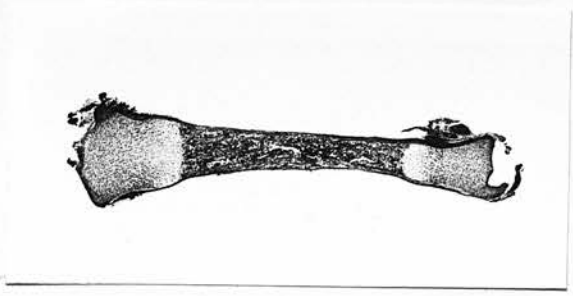
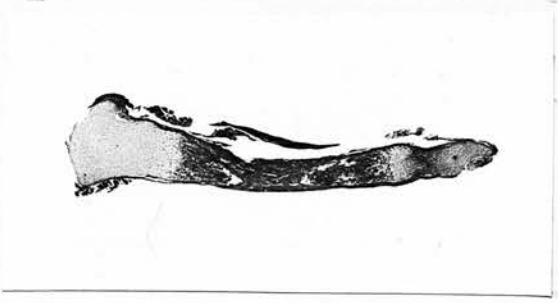


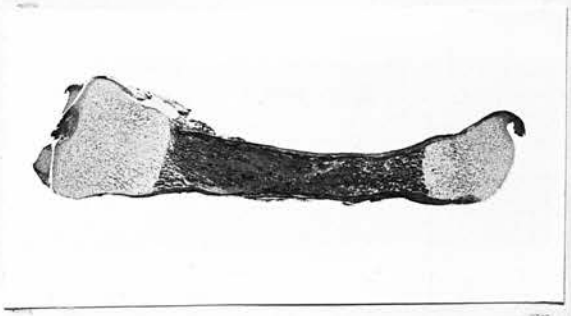
Fig. 40. Comparison of Growth in Length in Vivo and in Vitro

BONE	GROWTH	2 days	4 days	6 days
Tibia	in vitro	12.3 ± 2.4	18.8 ± 6.6	20.8 ± 1.5
	in vivo	20.6 ± 4.7	47.8 ± 14.2	62.7 ± 13.1
Radius	in vitro	9.0 ± 3.5	10.4 ± 3.4	9.6 ± 1.4
	in vivo	21.3 ± 6.7	35.9 ± 3.8	53.5 ± 7.0
Ulna	in vitro	9.5 ± 2.7	8.7 ± 2.9	7.5 ± 2.3
	in vivo	21.7 ± 3.5	36.1 ± 5.3	49.5 ± 9.1

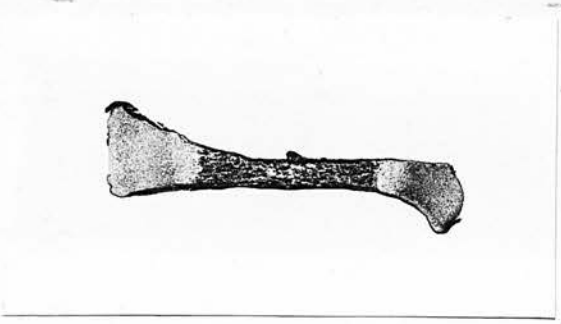


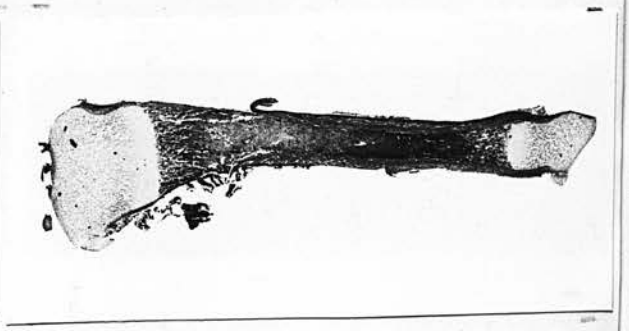
Control



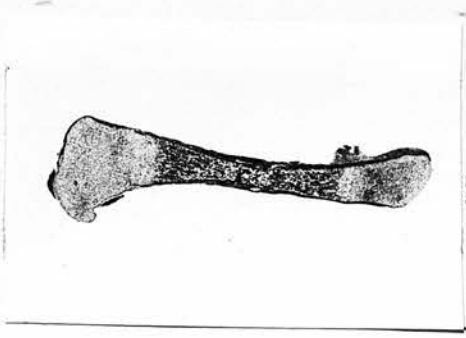


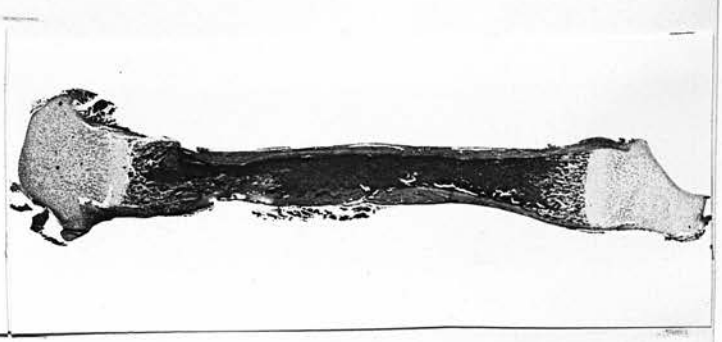
2 days In vitro In vivo





4 days In vitro In vivo

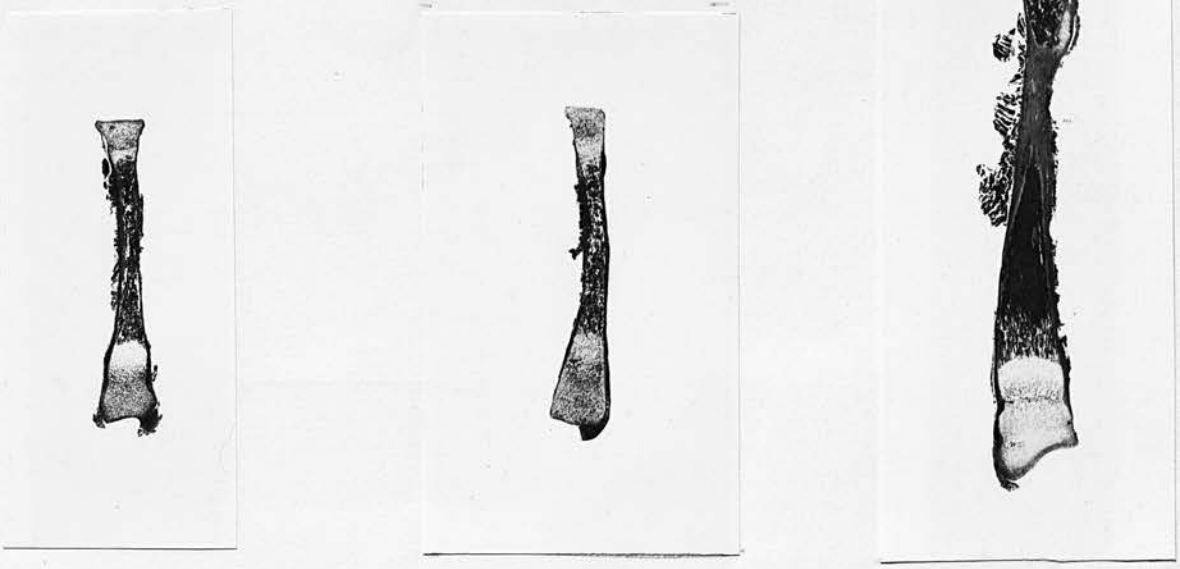




6 days In vitro In vivo

Alcian Blue & PAS x 13

Fig. 41. Comparison of Tibial growth in vivo and in vitro.



Control 6 days in vitro 6 days in vivo

Fig. 42. Comparison of Radius growth in vivo and in vitro

Control 6 days in vitro 6 days in vivo

Alcian blue & PAS x 3

Fig. 43. Comparison of Ulna growth in vivo and in vitro

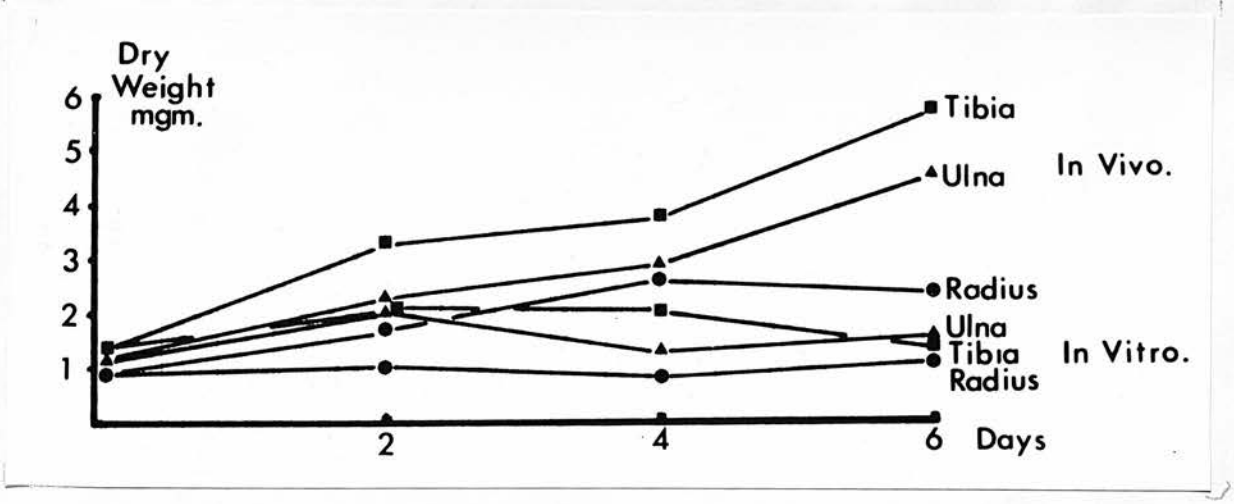
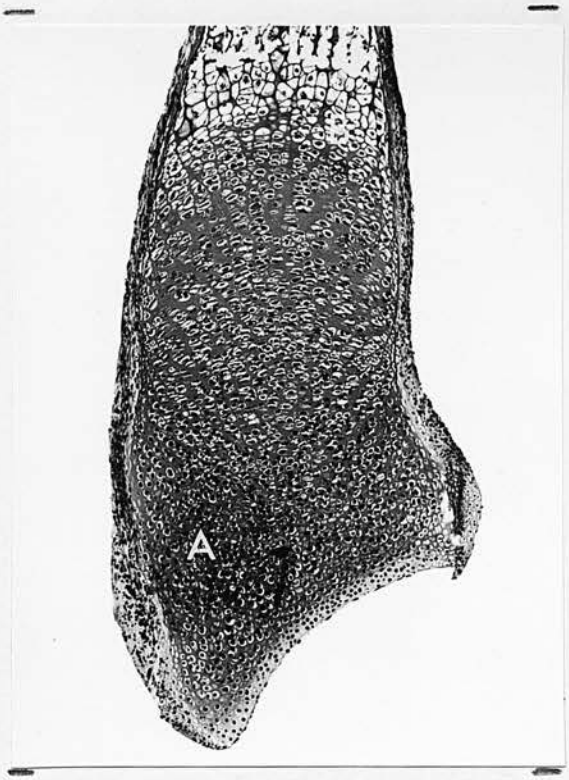


Fig. 44. Comparison of Dry Weight in vivo and in Vitro.

TABLE 6

BONE	CONDITIONS	TIME (DAYS	WET WEIGHT	DRY WEIGHT
Tibia	control	Zero	7.4	1.4
	in vitro	2	7.0	2.0
		4	10.4	2.0
		6	9.4	1.4
	in vivo	2	7.6	3.3
		4	19.3	3.8
		6	22.0	5.8
Radius	control	Zero	3.1	0.9
	in vitro	2	2.7	1.0
		4	3.0	0.8
		6	2.9	1.1
	in vivo	2	4.3	1.7
		4	6.2	2.6
		6	6.9	2.4
Ulna	control	Zero	4.8	1.2
	in vitro	2	4.9	2.0
		4	5.5	1.3
		6	5.4	1.6
	in vivo	2	7.2	2.3
		4	8.0	2.9
		6	10.6	4.6

N.B. Combined weights in mgms. of 2 rudiments.



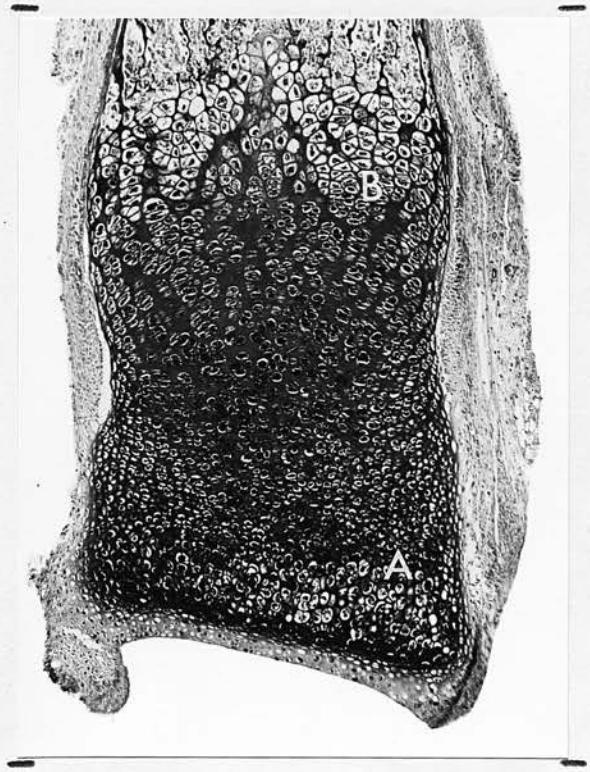
A. Positive PAS staining with chondrocyte hydration

B. Hypertrophic cells

Alcian Blue & PAS

x 80

Fig. 45. End cartilage of radius after 6 days in vitro



A. Positive PAS staining with chondrocyte hydration

B. Hypertrophic cells

Alcian Blue & PAS

x 80

Fig. 46. End cartilage of radius after 6 days in vivo

DISCUSSION

As expected there was relatively poor longitudinal growth of the limb bones in vitro when compared with that in the intact animal. With the optimum conditions for culture, determined in part I, the maximum achieved was 20% in the tibia and less for the other bones. The increase resulted from hypertrophy of the end cartilages, but there was no associated increase in wet weight to suggest overhydration, confirming the histological impression that this lengthening resulted from chondrocyte division in the proliferative zone.

The failure of continued growth in vitro by endochondral ossification must reflect the loss of the blood supply to the metaphyseal area. What is less clear is whether this vascular ingrowth in vivo is an active process resorbing chondrocytes from the distal portion of the hypertrophic zone, or whether it is a response to preceding degeneration in the cartilage. If this degeneration were the result of a low oxygen tension in the hypertrophic zone, comparison with the results of rudiment culture in higher oxygen tensions, to be investigated in part III, would be valuable. It is still possible that the process of bone formation on the surface of the cartilage cores is dependent on a constant supply of new osteoblasts arising from the endothelial cells of the capillaries as suggested by Trueta (1963). If this concept is correct, the creation of the ideal local gaseous environment in the metaphysis would still not restore bone formation. Another possible source of these bone forming cells was suggested by Holtrop (1966). By

prelabelling chondrocytes with tritiated thymidine she was able to demonstrate that transplants of cartilage, both in vivo and in vitro, could be made to form bone. As the label, previously present in the hypertrophic chondrocytes, was now seen in the osteoblasts and osteocytes it was suggested that the cartilage cells were precursors of osteogenic cells. To exclude the obvious mechanism of perichondrium conversion to periosteum, she stripped this layer from the explants, but its removal may have been incomplete. If her hypothesis is correct another cause of failure of endochondral ossification in vitro would be an arrest of chondrocyte production and maturation, which would accord with some of the histological findings.

The periosteal osteoblasts of the shaft continue to function in culture producing new osteoid on the surface to replace the endosteal resorption. Their survival compared with the disappearance of deeper metaphyseal osteoblasts may reflect the gradient of gas diffusion from the surface of the rudiment to the marrow cavity. Continued turnover of bone matrix at this site permits biochemical study of this activity in vitro, albeit on a limited basis.

The increase in dry weight of the bones in culture was also disappointingly small compared with the in vivo situation. In all three bones studied it reached a maximum at two days and thereafter declined returning to the control level. When taken with the time of maximum elongation this would suggest a progressive degeneration of rudiments in culture from the second day onwards. Despite this, no loss of dry weight was recorded, suggesting that either resorption was

balanced by continued synthesis, or that the resorptive mechanism was also inhibited in culture. As increase in length and dry weight during culture was greatest in the tibia, this bone was chosen for the remaining experiments in this study to provide the maximum quantity of tissue.

The changes in the end cartilages of the radius and ulna seen in vivo from the fourth day after birth should result in the formation of secondary ossification centres, if the time of appearance described by Johnson (1933) was correct. An increase in size and vacuolation of the epiphyseal chondrocytes, deposition of glycogen, and loss of metachromasia in the ground substance are the characteristic features preceding ossification in cartilage. The appearance of similar changes in the end cartilages of the bones in culture would suggest that the stimulus to the cells at this site is still active. If the development of the secondary ossification centres was merely a response to chondrocyte degeneration at the cartilage centre, it should be prevented by maintaining their viability. The degeneration may result from inadequate diffusion of nutrients or oxygen to the cartilage centre and if the latter, should be reversed by increasing the oxygen concentration in the gas phase. This feature will also be investigated in part III. The findings here would certainly suggest that the initial changes leading to the formation of the secondary ossification centre are not dependent on the metaphyseal blood supply. Nevertheless, this might be necessary for their complete development and viability could not be maintained for a sufficient time in culture to establish this point.

PART III

STRUCTURAL CHANGES INDUCED BY HYPEROXIA

INTRODUCTION

Some of the evidence describing the differing effects of hyperoxia on skeletal tissue in vivo and in vitro has been reviewed in the General Introduction. Experiments using oxygen micro-electrodes may give some indication of the local oxygen tension within bone and cartilage, but this may be altered by the local blood supply. Circulatory changes may also alter pH and carbon dioxide concentrations, which could modify the oxygen effect. In vitro culture represents a more artificial situation, and though the degree of hyperoxia can be accurately controlled, other factors in the complex nutrient media may exert an effect. Some of these differences have been described in Section I comparing growth on synthetic and natural media.

Goldhaber (1958) in his original observation on cultured mouse calvaria, showed that 95% oxygen markedly increased bone resorption and only in the later stages, after 10 or 11 days, was there some increase in osteoid formation. Though early resorption was marked, it was not associated with the appearance of typical giant cells of the osteoclast type until the third day. Much of this resorptive activity seemed to be related to macrophages, though these cells may later coalesce to form typical osteoclasts. He used a natural medium for his stationary cultures, thereby introducing the unknown biological activity of embryo extract and heparinised chicken plasma. The same worker (Goldhaber, 1965) showed that heparin, in as low a dosage as 0.2 units per ml., could act as a potent co-factor in the

resorption produced by parathyroid hormone and oxygen. When he used the roller tube method for culture of the calvaria, resorption was obtained at an even lower oxygen concentration of 20% (Goldhaber, 1963). Considerable variation in the extent of bone resorption was seen over a small spectrum of oxygen concentration, with even greater removal of bone in 30% oxygen. This resorptive effect of hyperoxia was partially inhibited by eliminating the embryo extract from the culture medium, suggesting that some unidentified factors were activated by the increased oxygen concentration.

When cartilaginous limb-bone rudiments from eight-day old chick embryos were exposed to hyperoxia, similar resorption was seen as the major feature, though there was some stimulation of periosteal bone formation (Sledge and Dingle, 1965). There was a loss of metachromasia in the cartilage ground substance, particularly in the hypertrophic zone, associated with softening and distortion of the rudiments, which failed to elongate normally. Collagen loss was less well marked and was associated with the presence of osteoclast-type cells, not normally present in chick bone at this age.

Shaw and Bassett (1967) took tibial explants from slightly older, eleven-day chick embryos and cultured them for two weeks in a range of oxygen concentrations from 5% to 95%. The most marked periosteal new bone formation was reported in 35% oxygen, although this was associated with loss of cartilage metachromasia at the cut ends of the rudiments from which the epiphyses had been removed. Some chondrocytes in this area showed alteration

in their lacunae, resembling osteoblasts, with occasional collagen fibres lying in the matrix in relation to them. In 95% oxygen less new bone was formed, though this still exceeded that in the air controls. There were larger lacunar spaces in the osteoid formed, with a significant increase in the number of cells per unit volume of extra-cellular matrix. These changes suggested a resorptive process, although multinucleated cells, resembling osteoclasts, were very scanty. The metachromasia was maintained in the cartilage ends and there were none of the alterations in cartilage cells seen with 35% oxygen. Migrating mesenchymal cells from the cut ends of rudiments showed definite evidence of chondroclastic activity.

It was suggested that these results demonstrated a spectrum of the effects of oxygen on bone and cartilage cells. High oxygen concentration produced osteoclasia and chondroclasia, while intermediate oxygen concentrations stimulated osteogenesis, and low oxygen concentrations resulted in minimum bone formation, but promoted cartilage formation.

These observations led Bassett (1962) to investigate the effects of hyperoxia on undifferentiated cellular mesenchymal tissue. He took bone fragments from twenty-day old chick tibiae and embedded them in a clot of embryo extract and chick plasma. At six days the implants were cut out from the ring of cellular outgrowth which they had produced and this was subjected to different degrees of oxygenation, as well as mechanical stimuli. He showed that in a high concentration of 95% oxygen the mixture

of polygonal and spindle cells passed through a transient phase when they resembled cartilage, but quickly became ossified with a surrounding calcified matrix. A few cartilage cells remained at the centre of the tissue, suggesting that the differentiation depended on an oxygen diffusion gradient from the periphery. It was concluded that cells behaved as chondroblasts or osteoblasts, depending on the availability of oxygen, and that this determined the form and composition of the extracellular matrix. In an attempt to rationalise these results with those of Goldhaber, who had reported bone resorption from hyperoxia, he suggested that cell function in culture is largely dependent on the micro-environment interacting with the state of cell specialisation prior to explantation.

Brighton and his co-workers have published the results of an elegant series of experiments with somewhat different conclusions on the effects of hyperoxia. They used a growth-plate model, the isolated costo-chondral junction of young rats, and exposed this in vitro to a range of oxygen concentrations. In their first report (Brighton et al., 1969) they showed that bone and cartilage growth were balanced in 5% oxygen, but that this occurred at only a fifth of the in vivo rate. At all higher concentrations cartilage growth exceeded bone growth, rising by a factor of four in 21% oxygen. At this concentration the amount of bone present at the bone-cartilage junction was equivalent to normal in vivo controls. In 90% oxygen an initial increase in the length of cartilage, possibly due to a failure of metaphyseal cell invasion, was followed by a decrease and

finally a disappearance of the hypertrophic cell layer. This was associated with a loss of metachromatic staining in the ground substance and an accumulation of PAS-positive material and debris at the bone-cartilage junction. The bony portion of the plate was resorbed, but no associated increase in osteoclasts was seen. They concluded that osteogenesis has a different lower optimum oxygen tension to chondrogenesis. In the intact animal probably neither process is operating under optimum conditions, even though the oxygen tension may be different on the two sides of the growth plate.

Brighton and Heppenstall (1971) extended these studies by recording the oxygen tensions in the zones of the growth plate, and the metaphysis and diaphysis, using platinum micro-electrodes. In vitro the tension was shown to be significantly higher on the bone side of the plate, when compared with the cartilage. This fell to a lower common level after ten days in culture, due to the loss of cell function preceding death. There was a distinct and increasing tension for each zone of the growth plate, rising to a maximum of 75 mm. Hg. in the hypertrophic cell layer. This did not parallel the findings in vivo, where the tension fell in the hypertrophic cell layer to 25 mm. Hg. It was also low in the metaphyseal bone at 20 mm., compared with the 95 mm. in vitro. They suggested that the abnormally high oxygen tension at the bone-cartilage junction was associated with the low growth rate, and explained why growth had been greater in low oxygen tensions. They felt that the descending gradation in oxygen tension through

the plate from the high level on the metaphyseal side represented oxygen diffusion, the intact hyaline cartilage being less pervious that the cut end of the bone. It was thought that the low oxygen tension in the hypertrophic cell layer and metaphysis in vivo represented a physiological mechanism to stimulate the rate of growth by anaerobic metabolism, rather than excess utilisation at this site. It also suggested that the release of lysosomal enzymes was not the normal mechanism for cell digestion, unless these were released by the oxygen gradient at the diaphysis-metaphysis junction by metaphyseal cells. The alternative explanation is that lysosomal enzymes in the hypertrophic layer were released in response to some stimulus other than hyperoxia. This might be a depression of mitochondrial enzyme activity in these cells, which is normally very high.

In one of the few studies using mouse limb bones, Ellis and Peart (1970) confirmed that heparin in a dose of 1.0 mg. per ml. of media produced increased bone resorption and impaired new bone formation. They also reported that air was the gaseous phase giving optimum bone growth and preservation and that 95% oxygen produced bone resorption. However, no details were given as to the site and mechanism of this resorption.

Studies of the effect of hyperoxia on isolated cells have been few and those reported have not included skeletal tissues. Allison (1965) showed that 95% oxygen was toxic for both chick embryo cells and mouse macrophages, producing rounding and vacuolation after 36 hours. These changes were associated with a release of lysosomal enzymes, demonstrated by acid phosphatase

staining in the cytoplasm, probably resulting from the increased permeability of lipo-protein membranes produced by excess oxygen.

Hyperbaric oxygen is known to be even more toxic for cells, but their sensitivity varies considerably. Halasz and Stier (1966) grew a wide range of adult tissues in organ culture exposed to hyperbaric oxygenation. The specialised tissues of brain, lung and thyroid showed early death, but lymph nodes, liver and spleen all showed good resistance and active mitosis. In addition to the peroxidation of lipid in cell membranes, the toxicity may be associated with interference with sulph-hydryl containing enzymes as a specific metabolic block. This depresses oxygen utilisation by tissues, probably not as a result of direct interference with glucose metabolism, but from a defect in pyruvate utilisation and high energy phosphate bone formation. The relative resistance of connective tissues might suggest that the effect of hyperbaric oxygen on bone and cartilage cells might not be totally destructive.

Reversibility of another toxic effect was reported by Race et al. (1969) when they exposed rat adrenal cortex to high pressure oxygen in vivo and in vitro. The main toxic effect occurred at an ultrastructural level in the cellular mitochondria. These became ballooned and degenerate, with associated vesicular degeneration of the endoplasmic reticulum. The changes were less widespread in the cells of the in vitro preparation, suggesting that the absence of ACTH in some way diminished the toxicity. Of greater interest was the recognition of repair of

the mitochondrial membranes within one to two hours following removal from the high pressure environment, demonstrating the reversibility of this effect. In this particular tissue it was thought that lysosomal activity did not contribute to the cell degeneration, although these might have played a part in other cells containing more of the sub-cellular bodies.

In the absence of relevant literature relating to the effects of hyperoxia on the structure of mammalian limb bone rudiments in culture, experiments were designed to provide answers to the following questions:-

1. Is the effect of hyperoxia on mammalian limb bones in vitro similar to that reported for avian rudiments?
2. What are the differences in tissue survival and growth when these rudiments are exposed to high oxygen concentrations at atmospheric and hyperbaric pressures?
3. Are there differences in the behaviour of cartilage and bone in these mixed rudiments?
4. Does endochondral bone formation continue in the absence of a circulation and how is it influenced by elevation of oxygen tension?
5. If bone and cartilage resorption occur with hyperoxia, what cellular changes accompany this process?

MATERIALS AND METHODS

1. Preparation of Explants.

Paired 'intermediate' or ' late' tibiae, obtained from 17 - 19 day old foetal mice of the Tuck's No. 1 strain, were used for all the experiments in this part of the study. The bone rudiments were prepared for explantation using the sterile technique described in part I of this thesis.

2. Culture Technique.

The culture technique described in part I was used for these experiments with three or four tibial rudiments to each grid and with 1.5 ml of medium in each small dish.

3. Culture Medium.

The P6 modification of BGJ medium was used in all the experiments, supplemented with 15% heat inactivated foetal calf serum. In a few experiments where the explants were studied by autoradiography tritiated proline was added to the medium in a dosage of 1.0 μci/ml. The medium was changed after each 48 hour period of culture and the pH checked. If this differed by more than $\pm$ 0.1 between cultures for comparison, or if it fell outside the physiological range, the results were discarded.

4. Gas Phase.

Three gas phases were used in this part of the study and experiments were designed to compare all three with rudiments from the same litter.

a) 95% air + 5% carbon dioxide - at atmospheric pressure.

b) 95% oxygen + 5% carbon dioxide - at atmospheric pressure.

c) 98% oxygen + 2% carbon dioxide - at 2 atmospheres absolute pressure (hyperbaric).

To achieve oxygenation at hyperbaric pressure a small experimental chamber supplied by Vickers Research Limited was used (Fig. 47). This was designed to accept the same petri-dish carrier as used in the anaerobic jars. The flow of gas from the cylinder reducing valve through the chamber could be controlled by entry and exit valves. After flushing the chamber for five minutes with the gas at atmospheric pressure the exit valve was closed to allow the pressure to rise slowly to the pre-set level as shown on the pressure gauge. The entry valve was left slightly open during the period of culture to compensate for any slight leaks from the chamber.

5. Methods of Examination.

a) Growth in Length.

Rudiments were measured at the time of explantation and every one to two days using the microscope eyepiece graticule.

b) Histology.

The rudiments were processed for histological examination after harvesting using the techniques of fixation, dehydration, embedding, sectioning, staining and mounting as described in part I.

c) Autoradiography.

In the experiments using medium labelled with tritiated proline the rudiments were fixed, processed and stained for autoradiographic examination by the techniques used in part I.

FIG. 47. EXPERIMENTAL HYPERBARIC CHAMBER

a) Chamber lid and pressure screws

b) Safety valve

c) Petri dish and carrier

d) Pressure gauge

e) Inlet valve control

f) Outlet valve control

RESULTS

The experiments were designed so that pairs of rudiments from the same animal and the same litter could be compared in each of the three gas phases. This allowed a direct comparison of the effect of variation in oxygen concentration and pressure.

1. Growth in length.

The percentage growth in length of eighteen pairs of 'intermediate' tibial rudiments was compared in the three gas phases over a six day period of in vitro culture. The results are shown graphically in Figure 48 and in Table 7. They show maximum elongation in air with figures similar to those obtained using BGJ medium in Part 1. The increase in length of rudiments exposed to hyperoxia was poor by comparison, barely half that obtained in air. After the first two days in culture there was no further increase in length of the rudiments in 95% oxygen or hyperbaric oxygen.

2. Histological changes.

The histological changes described were based on a study of sections from 69 pairs of tibial rudiments cultured in fourteen experiments. The serial changes produced by the various oxygen concentrations were similar between experiments with only small variations.

a) After one day in culture.

The end cartilages showed little difference except for a slight loss of the normal 'zoning' in rudiments exposed to hyperoxia. This probably resulted from increased vacuolation of the normally flattened cells of the proliferative layer.

TABLE 7

Effect of Gas Phase on % Increase in Length of Tibial Rudiments in Vitro.

GAS PHASE	TIME (days)	% INCREASE OF RUDIMENTS (12)	MEAN	S.D.
95% air + 5% CO_2	2	15.6, 13.8, 1.4, 10.3, 10.6, 8.7 8.5, 6.8, 8.6, 17.4, 2.6, 6.6	9.2	4.7
	4	4.3, 17.2, 18.0, 11.7, 12.1, 10.9 22.5, 7.4, 13.9, 19.0, 21.4, 10.5	14.1	5.6
	6	18.8, 6.9, 15.6, 15.3, 11.8, 12.1 9.7, 22.0, 22.7, 17.4, 22.8, 10.5	15.5	5.4
95% O_2 + 5% CO_2	2	4.9, 4.7, 10.8, 4.7, 2.5, 3.7 6.0, 4.5, 2.9, 1.3, 20.0, 5.5	5.9	5.0
	4	3.7, 6.2, 6.0, 6.3, 3.7, 3.7 6.2, 7.2, 5.0, 15.7, 2.6, 1.4	5.6	3.6
	6	2.5, 4.7, 4.8, 7.8, 1.2, 3.8 7.5, 6.0, 3.8, 21.4, 5.5, 2.7	6.0	5.2
98% O_2 + 2% CO_2 @ 2 ATS. (HBO)	2	2.4, 4.4, 1.4, 3.6, 5.1, 7.6 13.6, 9.4, 9.1, 4.1, 2.7, 1.4	5.4	3.7
	4	4.4, 1.4, 9.1, 6.1, 10.9, 9.1 1.4, 2.4, 7.7, 5.3, 4.1, 2.6	5.4	3.2
	6	4.4, 2.8, 5.6, 1.4, 3.0, 6.1 4.7, 10.6, 7.7, 2.6, 3.8, 2.4	4.6	2.6

The most dramatic effect was seen in the metaphyseal zone of endochondral ossification, where numerous osteoclasts were actively resorbing newly formed osteoid and degenerate cartilage in rudiments grown in 95% oxygen (Fig. 49A). By contrast rudiments in hyperbaric oxygen and air showed no or minimal resorption and only occasional osteoclasts (Fig. 49B and 49C).

The bone of the shaft was well preserved in air and the periosteal osteoblasts and deeper osteocytes were healthy and numerous showing a very active tritiated proline label incorporation into the osteoid (Fig. 50A and B). The periosteal osteoblasts in rudiments exposed to hyperbaric oxygen were still numerous but appeared more spindle shaped or flattened, with a few examples of nuclear pyknosis or fragmentation visible (Fig.51A). Despite this the labelled proline was still incorporated into the collagen of the bone on autoradiography, though not as heavily as in air (Fig. 51B). The bony shaft appeared least healthy in 95% oxygen, with a thin or absent periosteal osteoblast layer and loss of more than half of the deep osteocytes (Fig. 52A). Light radioactive labelling was still visible in the superficial layers of the osteoid, but the well marked endosteal line seen in air and hyperbaric oxygen was largely lost (Fig. 52B). The marrow cells were diminished in number and size, though a few red cells were still present in all rudiments. The loss of the normal marrow population was much greater in both conditions of hyperoxia.

b) After two days in culture.

The end cartilages were still similar in appearance, though the broadening of the proliferative cell layer in air was more marked (Figs. 53A, B and C). In 95% oxygen the resorption in the metaphyseal area extended into the distal cells of the hypertrophic cell layer. The metachromatic staining of the cartilage ground substance was preserved except for a small central area at the junction of proliferative and hypertrophic zones in the air rudiments, where it was less dense in association with local chondrocyte degeneration. Tritiated proline label was diffusely present throughout the ground substance of the end cartilages, but was heaviest in the proliferative cell zone, particularly in air (Figs. 54A, B and C).

The rudiments grown in 95% oxygen showed a large gap between the hypertrophic cartilage cells and the remains of the trabecular bone due to the resorption of the endochondral bone. However, the majority of the osteoclasts which were responsible were now absent and only a few mononuclear scavenger cells remained (Fig. 55). There was still only minimal resorption at this site in the other two gas phases, though in air some osteoblasts were beginning to lay down osteoid on the surface of the distal layer of hypertrophic chondrocytes with positive azan staining (Fig. 56).

The bone of the shaft remained healthy in the air with preservation of periosteal osteoblasts and osteocytes showing some formation of new osteoid. In 95% oxygen the osteoblasts

were sparse and most of the deep osteocytes were lost leaving enlarged but empty lacunae (Fig. 55). In hyperbaric oxygen the osteoblast layer was thicker than with 95% oxygen and more osteocytes were retained, but both types of cell showed small, dense or fragmented nuclei suggesting death had occurred (Fig. 57).

The marrow cells continued to decrease in number in all the rudiments and only a few small mononuclear cells remained in the rudiments exposed to hyperoxia.

c) After four days in culture.

The end cartilages now showed distinct differences with the gas phase used. In air the general structure was maintained, though the cells in the proliferative layer were more rounded and a greater number of distal chondrocytes in the hypertrophic layer had lost their nuclei. In some rudiments perichondral cells were invading the cartilage in the junctional zone between proliferative and hypertrophic chondrocytes with an associated loss of metachromatic staining. In 95% oxygen all the chondrocytes now appeared degenerate with nuclear loss in about 50% of the cells in the proliferative zone and 80% in the hypertrophic zone. This was associated with a patchy loss of metachromatic staining in all layers and thinning of the perichondrium. By contrast rudiments exposed to hyperbaric oxygen showed preservation of more normal cell outlines, metachromatic staining and perichondrium thickness. However, nearly all the cell nuclei were small and stained densely suggesting death had occurred.

In the region of endochondral bone formation the air rudiments showed slight increase in cartilage and osteoid resorption though this was not associated with osteoclast proliferation. In

oxygen the cavity between cartilage and shaft bone had enlarged slightly, but in hyperbaric oxygen the resorption had not increased.

The shaft bone structure was maintained in air except for slight thinning of the periosteal layer and the new osteoid layer was as thick as the original bone (Fig. 58). In normobaric oxygen the bone had largely disintegrated from enlargement of the osteocyte lacunae, none of which contained osteocytes. The periosteum was absent or thinned with only flattened fibroblastic cells visible. In hyperbaric oxygen the osteocytes were largely lost or showed evidence of nuclear death but their lacunae were not enlarged to the same extent. The periosteal layer was also better preserved, though nearly all the cells showed pyknosis or fragmentation of their nuclei, resulting in a more normal bone architecture. In all rudiments exposed to hyperoxia the marrow cells were almost totally lost, but some mixed round cells persisted in air.

d) After six days in culture.

The degeneration of the end cartilages with both types of hyperoxia was even more advanced. As at four days, loss of nuclei and metachromatic staining was more marked in the distal zones than in the proximal epiphyseal zone. The distal end cartilage of the air rudiments frequently showed the development of a transverse layer of flattened cells continuous with the perichondrium overlying. This effectively divided the hypertrophic cells into two zones and was associated with positive azan and PAS staining suggesting new collagen formation (Fig. 59). This was

confirmed by an increase in tritiated proline labelling at this site on autoradiography.

In the metaphyseal region the air rudiments still showed a single layer of osteoblastic cells sealing the distal hypertrophic chondrocytes from the shaft. In some of the rudiments grown in 95% oxygen a similar 'seal' was present, but this was largely acellular and consisted of PAS-positive material. The appearances were otherwise similar to the four day rudiments.

The shaft bone showed preservation of periosteal osteoblast activity in air rudiments, confirmed by autoradiography which showed new label being incorporated at the surface of the osteoid as well as extending through its thickness. In a few rudiments endosteal osteoblasts were also active in new bone matrix formation (Fig. 60). No activity remained in the bone cells of the rudiments exposed to hyperoxia, though the original periosteal and endosteal ^{3}H-proline label was preserved in hyperbaric oxygen but largely resorbed in 95% oxygen. Widespread bone loss was evident in all the rudiments exposed to hyperoxia with a tendency to buckle and fragment on sectioning.

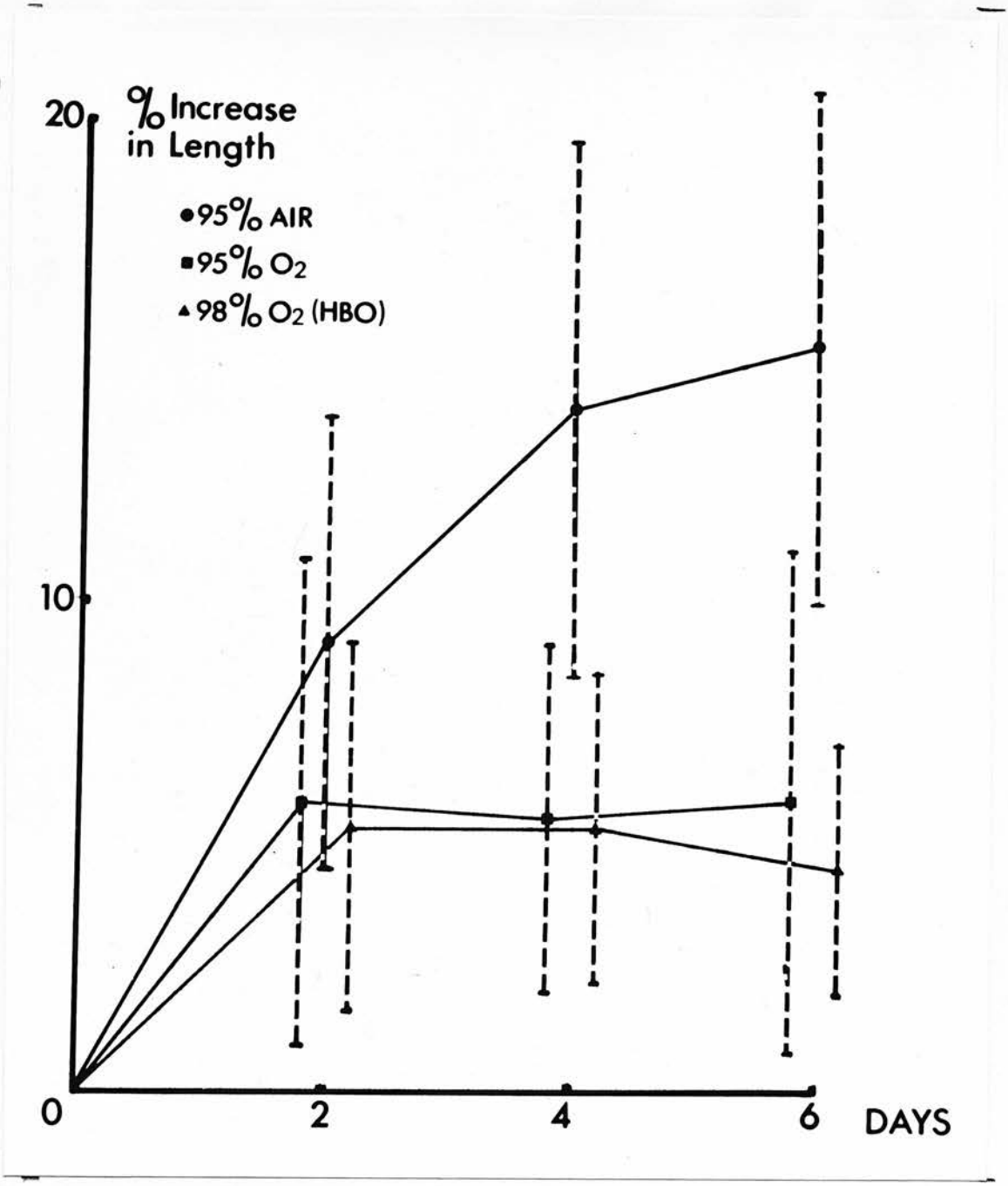
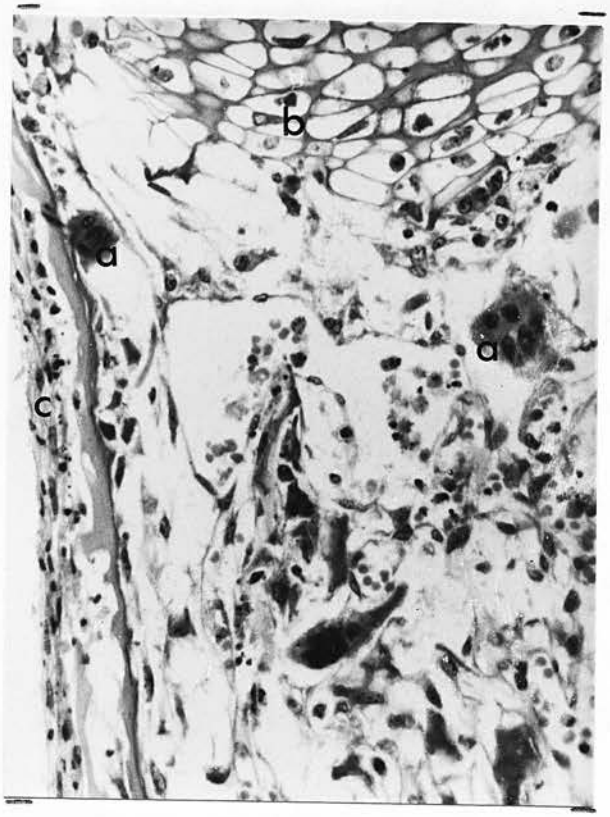


Fig. 48. Effect of Gas Phase on Growth in Length of Tibia in Vitro.



a. Osteoclasts

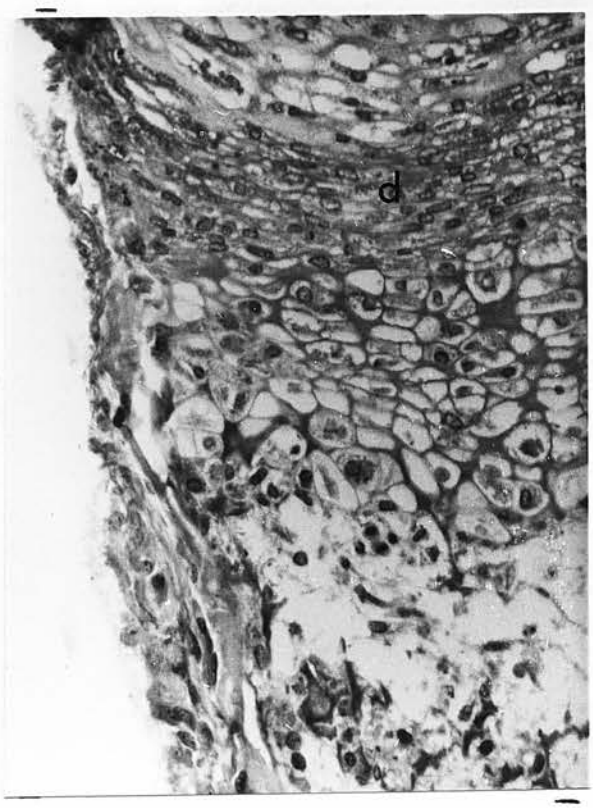
b. Hypertrophic chondrocytes

c. Periosteum

d. 'Flattened' chondrocytes

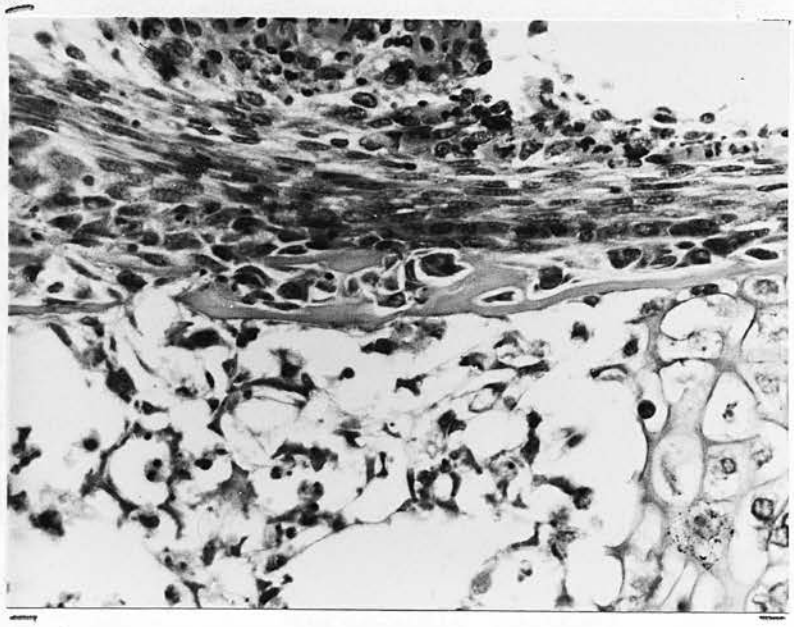
A. 95% Oxygen

B. Hyperbaric Oxygen



C. 95% Air.

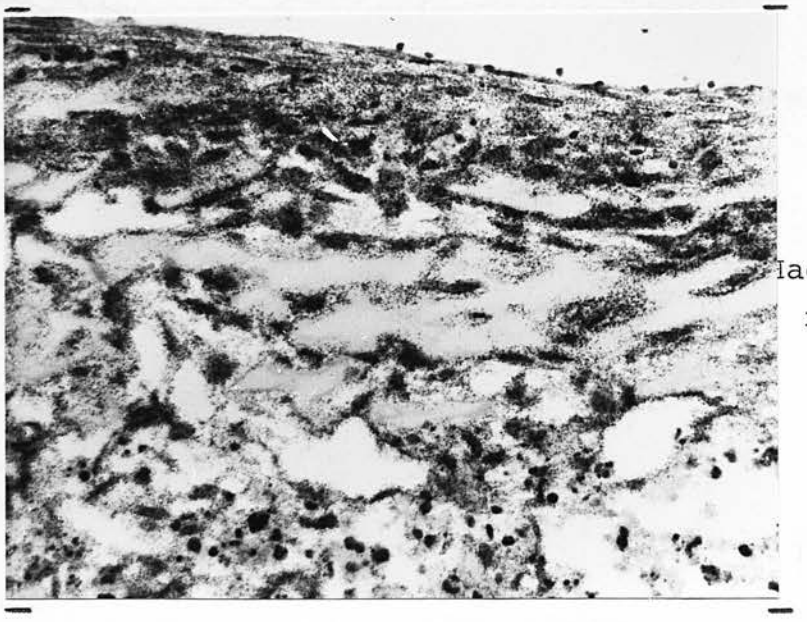
Figs. 49. METAPHYSEAL REGION AFTER 1 DAY IN CULTURE.
Alcian Blue and PAS x 240.



Alcian Blue and PAS
x 240.

Fig. 50A. SHAFT BONE AFTER 1 DAY IN AIR.

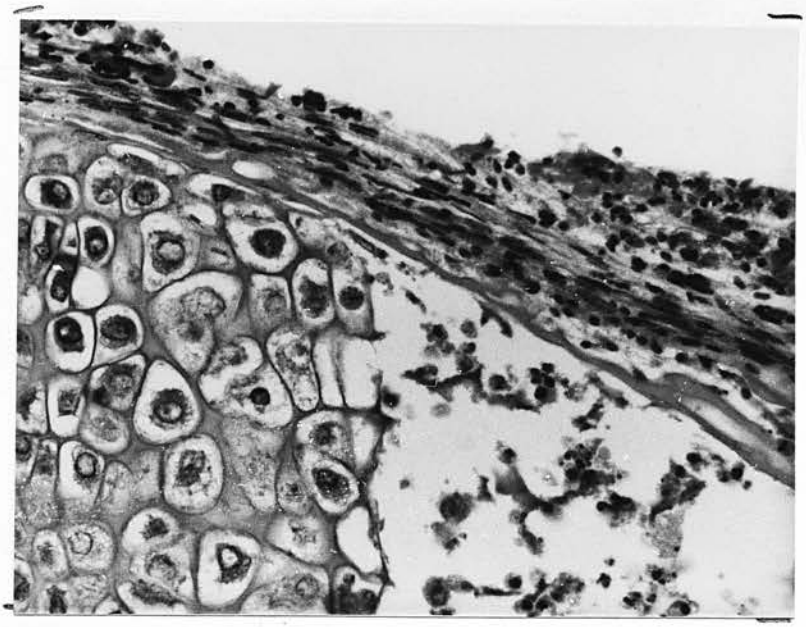
To show thickening of periosteal osteoblast layer.



Haematoxylin
x 240

Fig. 50B. AUTORADIOGRAPH OF BONY SHAFT AFTER 1 DAY IN AIR.

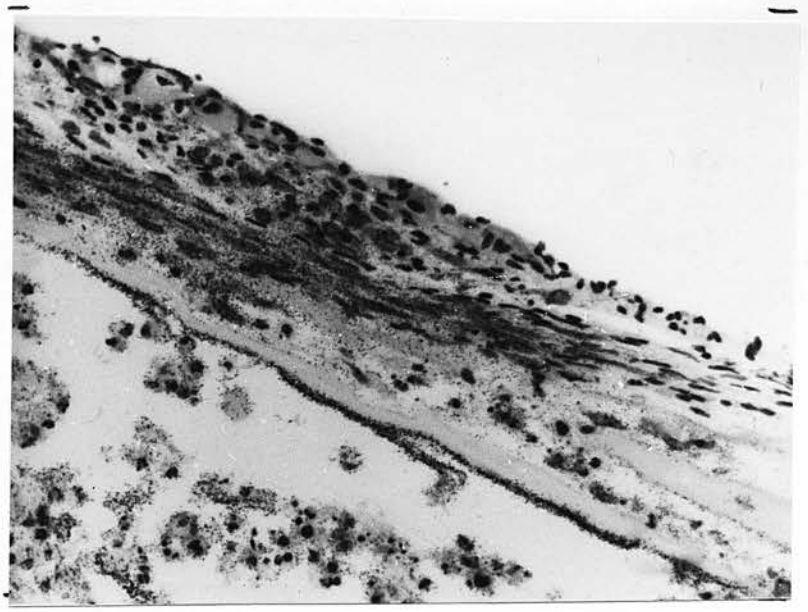
To show heavy labelling in periosteum and lighter label around trabecular bone.



Alcian Blue and PAS
x 240.

Fig. 51A. SHAFT BONE AT METAPHYSIS AFTER 1 DAY IN HYPERBARIC OXYGEN.

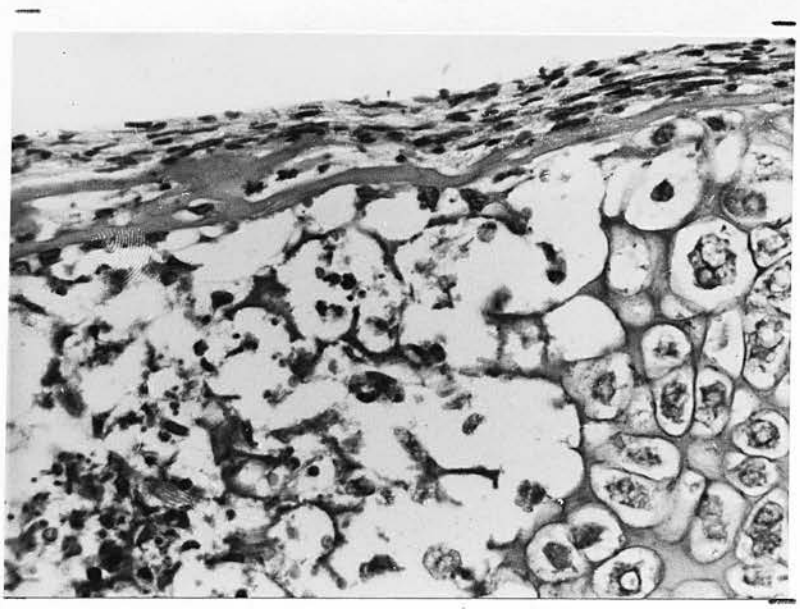
To show slight thickening of periosteal layer with nuclear pyknosis.



Haematoxylin
x 240.

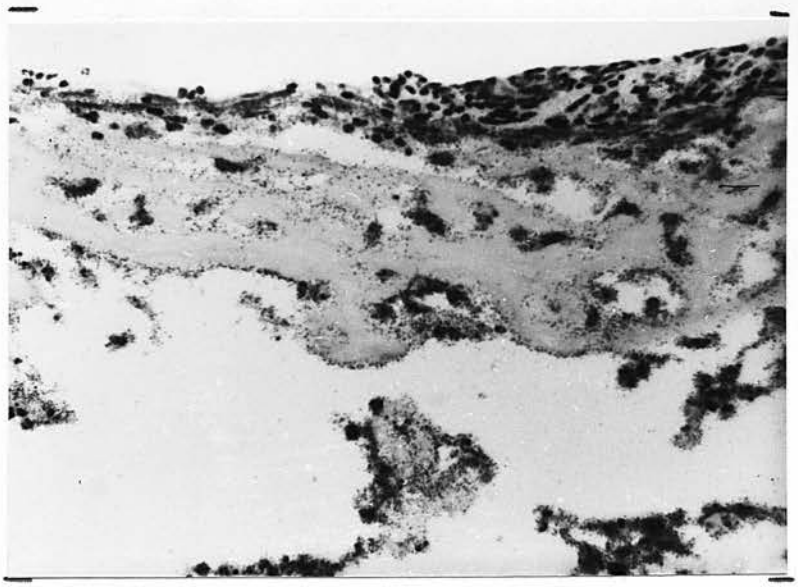
Fig. 51B. AUTORADIOGRAPH OF BONY SHAFT AFTER 1 DAY IN HYPERBARIC OXYGEN.

To show light labelling in deeper layers of periosteum and on endosteal surface.



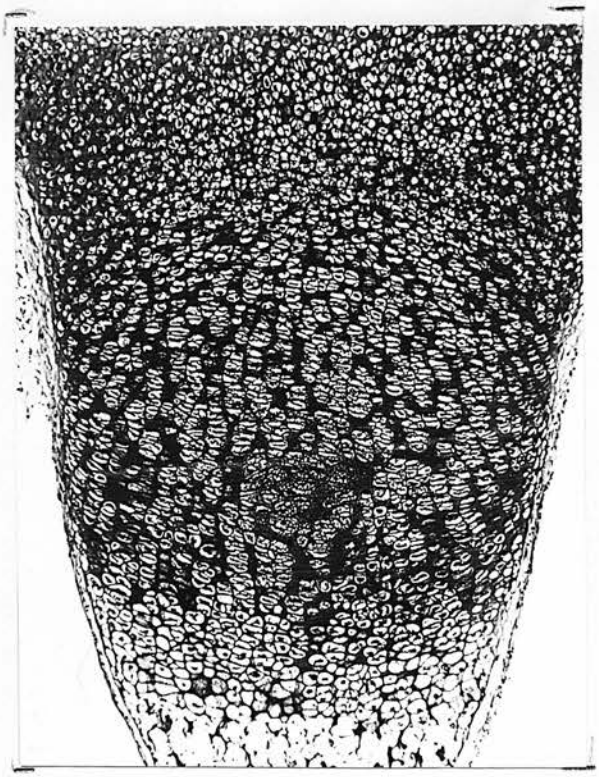
Alcian Blue and PAS
x 240.

Fig. 52A. SHAFT BONE AT METAPHYSIS AFTER 1 DAY IN OXYGEN.
To show absence of periosteal thickening and early osteocyte osteolysis.



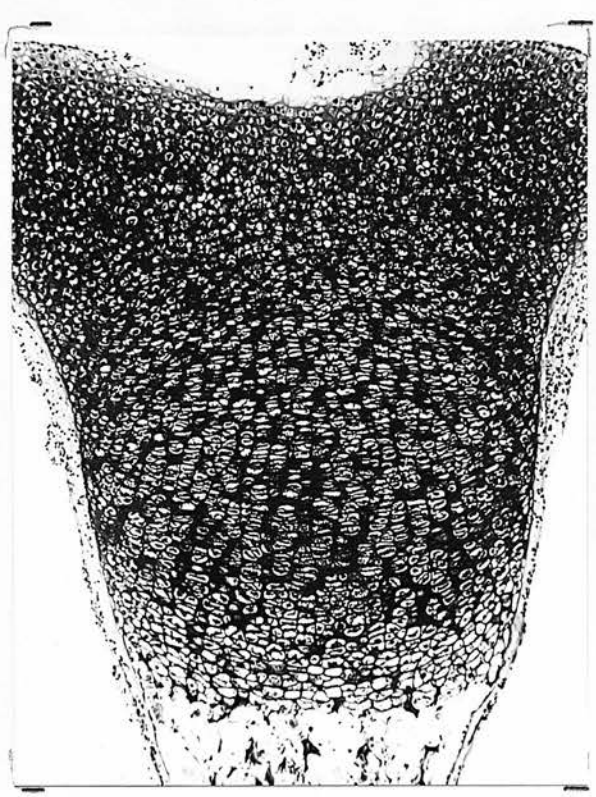
Haematoxylin
x 240.

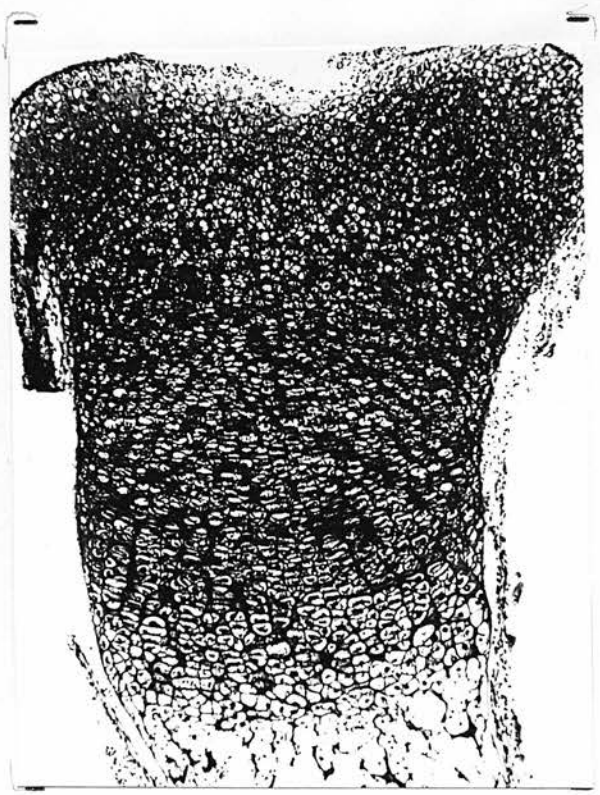
Fig. 52B. AUTORADIOGRAPH OF BONY SHAFT AFTER 1 DAY IN OXYGEN.
To show poor periosteal labelling and resorption of label from endosteal and trabecular bone.



Toluidine Blue
x 110.

A.





B.

C.

Fig. 53. END CARTILAGES AFTER 2 DAYS IN CULTURE.

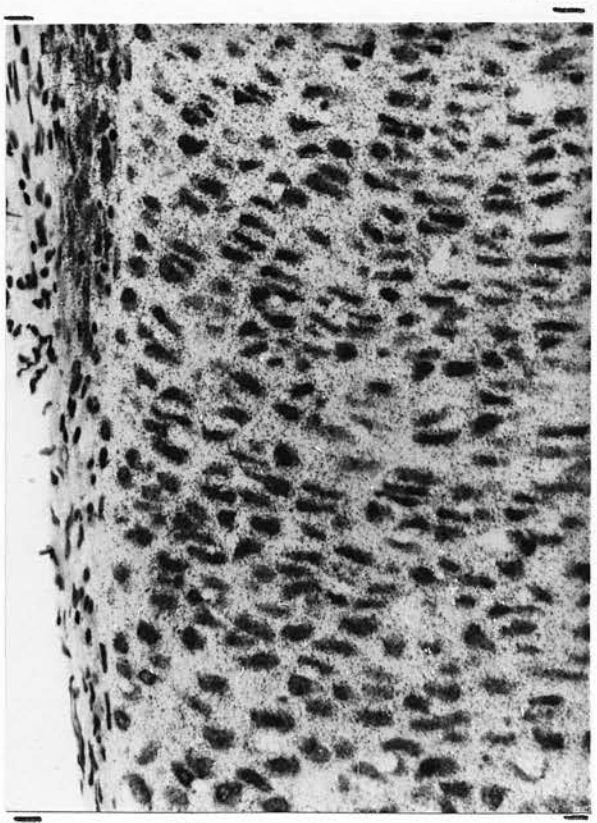
A. - AIR

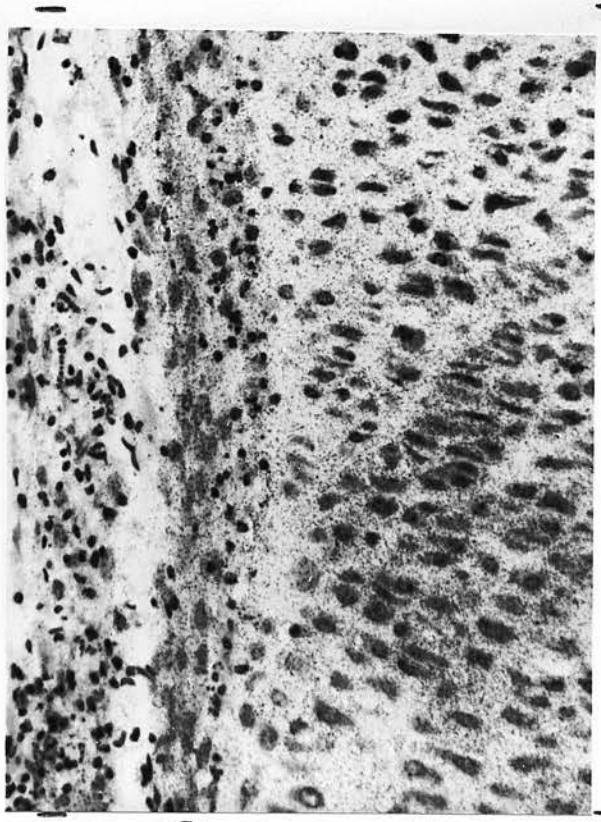
B. - OXYGEN

C. - HYPERBARIC OXYGEN

Haemotoxylin
x 240.

A.





B.

C.

Fig. 54. AUTORADIOGRAPHY OF END CARTILAGES AFTER 2 DAYS IN CULTURE.

A. - AIR
B. - OXYGEN
C. - HYPERBARIC OXYGEN

a. Osteoclasts

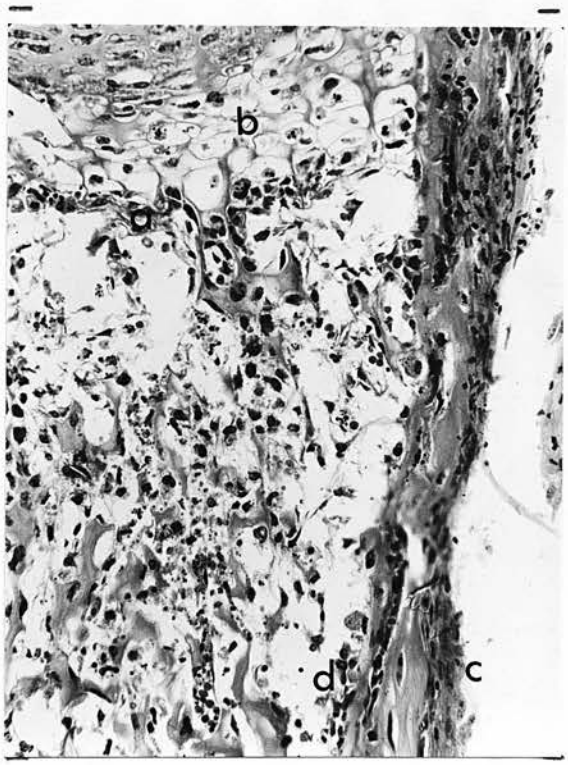
b. Hypertrophic chondrocytes

c. Periosteum

d. Enlarged osteocytic lacunae

H. & E. x 250

Fig. 55. Metaphysis after 2 days in 95% oxygen.



a. Osteoblasts sealing cartilage

b. Hypertrophic chondrocytes

c. Periosteum

d. Endosteum

H. & E. x 250.

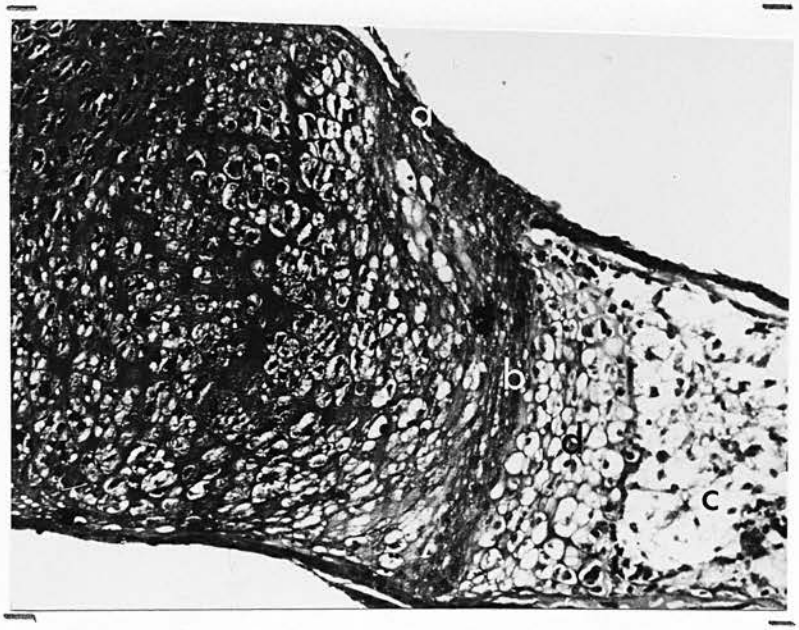
Fig. 56. Metaphysis after 2 days in 95% air.

a. Periosteal osteoblasts
b. Shaft bone with osteocytes
c. Cartilaginous trabeculae
d. Hypertrophic cells

Fig. 57. Shaft bone at metaphysis after 2 days in hyperbaric oxygen. H. & E. x 250.

a. New periosteal osteoid
b. Old periosteal bone
c. Hypertrophic cells

Fig. 58. Shaft bone after 4 days in air. H. & E. x 250.



a. Perichondrium

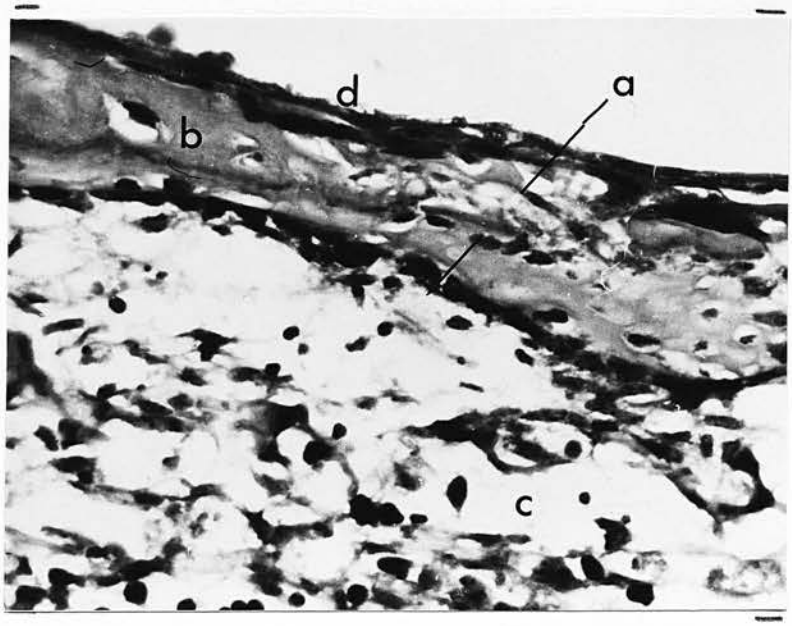
b. Flattened cell layer

c. Marrow cavity

d. Hypertrophic cells

Fig. 59. End Cartilage after 6 days in 95% Air.

Alcian Blue and PAS x 95.



a. Endosteal osteoblasts

b. Bone

c. Marrow cavity

d. Periosteum

Fig. 60. Shaft Bone after 6 days in 95% Air.

Alcian Blue and PAS x 375.

DISCUSSION

1) Comparison of hyperoxia in mammalion and avian limb bones in vitro.

The changes that were observed in the embryonic mouse tibia exposed to hyperoxia correspond in general to those reported with avian rudiments. With the high levels of oxygenation used, at both atmospheric and hyperbaric pressures, the rudiments all showed progressive degeneration and widespread necrosis. There were very few viable cells present in the rudiments after four days in culture and those that did survive consisted largely of undifferentiated fibroblast like cells. Loss of cartilage metachromasia was seen with 95% oxygen occurring patchly in the end cartilages, but it was not as complete or widespread as reported by Sledge and Dingle (1965) in the chick tibia. They used the rudiments at an earlier stage of development without a bony shaft, which might account for the difference since this was the site of major resorption in this study. The stimulation of periosteal bone formation, which was also reported in avian rudiments, was seen transitorily after one day in culture, but was quickly overtaken by the resorptive changes. As Shaw and Bassett (1967) reported the optimum oxygen level for this osteogenesis to be 35%, it might indicate that a more sustained stimulatory effect could be obtained in future experiments by the use of this lower concentration. The enlargement of osteocytic lacunae seen in these experiments with 95% oxygen would seem to correspond to the enlarged lacunae reported by Shaw and Bassett using the same oxygen tension. They were unable to identify osteoclasts in their material but as rudiments were grown for two weeks before

histological examination this is not surprising. These multinucleate cells were only seen in the mammalian rudiments in the first 1 - 2 days and thereafter disappeared.

2) Comparison of hyperoxia at atmospheric and hyperbaric pressures.

The effects of a high oxygen concentration in the gaseous phase were quite different at atmospheric and hyperbaric pressures. The resorption of bone and cartilage in the region of endochondral ossification was not so marked with hyperbaric oxygen and was not accompanied by the appearance of such a large number of osteoclasts. This together with the progressive nuclear death and fragmentation of both bone and cartilage cells suggests that the toxic effects of hyperbaric oxygenation kill these cells before resorption can occur. As the marrow cell population also showed early depletion mononuclear macrophages would also be lost. The osteocyte death seen with both types of hyperoxia was accompanied by more autolysis and lacunar enlargement at atmospheric pressure, suggesting that they were releasing proteolytic and other enzymes locally. Again the toxic effects of hyperbaric oxygen seem to prevent this process. The stimulatory effect of hyperoxia on periosteal osteoblasts was not evident in these results, although some thickening occurred transitorily under hyperbaric conditions. This did not continue after one day in vitro and would suggest that the toxic effects then supervene. As the stimulatory effect was not seen with normobaric oxygen it could be suggested that the increased pressure alone was responsible for the periosteal thickening. This would accord with the experimental results of Bassett (1964), who showed that compaction of undifferentiated mesenchymal cells led to bone formation, while tension induced fibrous tissue.

The results from autoradiography confirmed the transient production of new collagen in hyperoxia by osteoblastic cells. Much of this label was resorbed in ambient hyperoxia but was preserved in both endosteum and periosteum under hyperbaric conditions. It also persisted in the matrix surrounding osteocytes confirming that this is only resorbed in normobaric hyperoxia. Although the end cartilages showed patchy loss of ground substance metachromasia with 95% oxygen, it was preserved with hyperbaric oxygen suggesting a cell mediated enzyme system was responsible. In both conditions the tritiated proline label in the cartilage was preserved suggesting that collagenases were not primarily responsible.

What remains uncertain is whether a low oxygen tension of 35% would induce more bone formation and less resorption. It is possible that the toxic effects of hyperbaric oxygen might be reversible, as suggested by Race et al (1969), at least in the early stages, and that intermittent exposures would stimulate new bone formation without death. This might permit the renewal of endogenous DNA precursors, the production of which is thought to be disturbed by tissue hyperoxia.

3) Differing behaviour of cartilage and bone in hyperoxia.

The results suggest that bone may be more sensitive than cartilage to hyperoxia, though after exposure for two days both tissues are affected. The more undifferentiated cells showed a greater resistance to the toxic effects, particularly in the cartilage where the proximal epiphyseal chondrocytes survived to the late stages of culture. The hypertrophic cells showed more

resorption in hyperoxia, but this could be the result of increased chondroclast and osteoclast activity in the metaphyseal region, rather than autolysis of the cells themselves. The chondrocytes of the proliferative zone did not show the continued division seen in air, suggesting that oxygen inhibited their ability to reproduce. This failure would explain the poor growth in length of the rudiments under these conditions. The observed results support the concept of Brighton et al (1969) that hyperoxia enhances aerobic metabolism and inhibits development of the cartilage portion of the growth plate. The deposition of PAS-positive neutral polysaccharide debris sealing off the distal row of hypertrophic cells from the shaft was also observed by these workers. They attributed this to oxygen toxicity depressing mitochondrial enzyme activity in the hypertrophic cells, but equally it could result from the lack of capillaries or resorptive multinucleate cells to remove the material when formed.

The effects observed in the bone did not accord with the evidence suggesting that hyperoxia stimulates bone formation. However, most workers have reported optimum concentrations around 35%, which is much lower than those used here. It would fit with the concept of a spectrum of effects in bone, ranging from stimulatory in the lower concentrations to resorptive in the higher tensions. The initial stimulus to the periosteal osteoblasts seen at one day and later followed by suppression was also reported in vivo by Manspeizer and Tonna (1967) when mice were exposed to 100% oxygen. The effect was seen in the intact periosteum of the femur and was attributed to depletion of endogenous DNA precursors, a mechanism which would also operate

in vitro. It seems unlikely that periosteal bone formation would resume in these degenerate rudiments, even if the period of culture was extended to the 10 days reported as necessary by Goldhaber (1958). The survival of a few flattened fibroblast like cells in the periosteum and small round cells in the marrow confirms the greater sensitivity of differentiated cells to hyperoxia.

4) Endochondral bone formation and hyperoxia.

The histological studies on the rudiments grown in 95% air showed that endochondral bone formation can continue in the absence of a circulation. Autoradiography confirmed that labelled collagen was formed in the PAS-positive material laid down around the cores of degenerate cartilage in the metaphyseal region. This was assumed to be ostoid, though its degree of calcification is uncertain from these studies. It had extended to the entire shaft after 6 days and was also produced by the cells, showing the characteristics of osteoblasts, which sealed the distal hypertrophic cell layer. What was lacking seemed to be the orderly sequence of formation and resorption produced by the presence of a capillary circulation and the gradation of oxygen tensions along the shaft.

Brighton and Heppenstall (1971) showed that the oxygen tension is low in the metaphyseal region and rises to a high level in the diaphysis in vivo. This would suggest that endochondral bone formation required a lower oxygen tension than in the periosteum and would explain the early resorption seen in this zone with hyperoxia and the failure of further ossification on the remaining cartilage. The early development of the secondary ossification centre seen in the air rudiments did not occur in

either condition of hyperoxia. This was probably the result of failure of the perichondral cells to proliferate and invade the area of degeneration in the end cartilages.

5) Cellular changes accompanying resorption in hyperoxia.

Two distinct cellular processes accompanied the resorption of bone induced by hyperoxia. The first was associated with the appearance of osteoclasts, which actively resorbed cartilage and bone in the metaphysis. These could easily be overlooked unless the histology were studied in the early stages of culture since they largely disappeared after the second 24 hours. They were not seen in any great number with hyperbaric oxygenation suggesting that this in some way inhibited this cellular differentiation. It was not clear why their number fell off after the second day in culture and why further resorption by this mechanism ceased. Any explanation must be theoretical since the source of the osteoclasts themselves is uncertain. Whether they arise from fusion of osteoblasts or differentiate from mesenchymal cells, it is known that they are extremely active in RNA synthesis (Owen, 1967) and enzyme production (Hancox and Boothroyd, 1963). If oxygen acts as a stimulus to their production it may also poison their enzyme systems or the production of DNA precursors.

The second mechanism of resorption was seen in the periosteal bone collar of the shaft and was associated with enlargement of the osteocytic lacunae. This process of osteocytic osteolysis and the stimuli which produce it has been reviewed by Belanger (1969). He felt that it was an active process and not a passive autolysis associated with a dying cell. This

would fit the observation that enlargement of the lacunae was less marked in hyperbaric oxygen, which must prevent the release of the resorptive enzymes by the cell. The increased PAS staining of the matrix surrounding these cells reported by Heller-Steinberg (1951) was not seen in these experiments. However, the bone matrix was normally strongly PAS-positive, even in the late stages of resorption when azan staining for collagen was decreased. From the electron microscopy studies of Belanger the osteocyte is known to contain lysosomes and as oxygen is a known stimulus to the release of their enzymes in other cells, it could also activate this resorptive mechanism.

Although hyperoxia at ambient pressure has been shown to induce bone resorption by these two mechanisms, the question remains as to whether this represents a physiological mechanism in the intact animal.

PART IV

COLLAGEN METABOLISM IN HYPEROXIA

INTRODUCTION

Metabolism of Collagen.

Collagen is a fibrous protein occurring extensively in all animal connective tissues. It forms over 80% of the organic matrix of bone and 55% of cartilage by dry weight. The lower content in cartilage results from the presence of non-collagenous proteins combined with polysaccharides to form more of the intercellular ground substance. In investigating the synthesis of collagen and its degredation in tissue resorption, use can be made of some of its unique chemical properties. It has a very high content of the amino-acids, glycine and proline, enabling these to be used for radioactive tracer studies, both by autoradiography and radiochemical methods. The fact that hydroxyproline specifically occurs in collagen and no other protein is also used. Hydroxyproline differs from proline in being synthesised in animal tissues by hydroxylation of proline residues in a polypeptide chain and not as a free amino-acid. Its degredation fate is also distinct, since its only known origin in connective tissue is from breakdown of collagen. The content of hydroxyproline can be estimated biochemically and converted to total collagen content using a known factor, which is species constant. In the case of mammals this constant is 6.94 (i.e. there is 14.4 gm of hydroxyproline per 100 gm of collagen).

Amino-acids present in a synthetic tissue culture medium may be incorporated into rudiments by the formation of collagen during the period of growth. At the same time the degredation of both old and newly-formed collagen within the rudiments may liberate

amino-acids into the culture medium. If a known quantity of proline, labelled with radioactive tritium, is used in the medium, the amount incorporated into the rudiments as new collagen can be determined by biochemical and radioactive analysis after hydrolysis Similarly any collagen resorbed during the period of growth will release hydroxyproline into the medium where it can be estimated biochemically. The proportion of this medium hydroxyproline showing radioactivity will indicate how much is liberated from newly-formed collagen and how much from old.

Biochemical estimation of hydroxyproline.

Before hydroxyproline can be estimated, the amino-acid must be liberated from collagen or polypeptides by hydrolysis. This is achieved most effectively by strong acidic conditions; the original method of Neuman and Logan (1950) autoclaved the tissue in 6N hydrochloric acid for three hours at 140^{o}C. If lower temperatures are used a longer period is required to produce satisfactory hydrolysis; three hours at 138^{o}C, or 24 hours at 100^{o}C (Jackson, 1967).

Colorimetric methods are based on the pyrrole reaction and have been reviewed by Jackson (1967). Hydroxyproline is oxidised to a pyrrole compound, which will then react with p-dimethyl-aminobenzaldehyde (P.A.B.) giving a red chromogen which can be read in the colorimeter at 560 m. The oxidants most commonly used are hydrogen peroxide or chloramine T. Hydrogen peroxide was used in the first practical method of Neuman and Logan (1950), but this gives poorly reproduced results because of interference with the coupling reaction and depressed colour yield if any of

the oxidant persists. The specific chromogen is also unstable in strong mineral acid and its colour tends to fade. For these reasons Stegemann (1958) used chloramine T (p-toluenesulphon-chloramide) as the oxidant in his method, as any excess is decomposed by the acidification step in the coupling reaction.

A variation of the Stegemann method, introduced by Bergman and Loxley (1963) was used in these experiments. Their methods were designed to minimise variation in colour yield and to make it more independent of hydroxyproline concentration over a wider range. They described two techniques; a rapid one carried out at 60°C, and a slow overnight one more suitable for the low hydroxyproline concentrations to be expected in this tissue culture system. The main modifications were to use isopropanol as an organic solvent instead of an aqueous solution and to increase the concentration of perchloric acid in the Ehrlich's reagent (P.A.B.). Less accurate timing was required and the method was carried out at room temperature giving satisfactory results with hydroxyproline concentrations between 2 and 15 μgms/ml.

The presence of other amino-acids from the tissue proteins may interfere with the colour reaction by producing chromogens themselves, particularly when hydroxyproline is present in relatively small amounts. This problem may be solved by using a modified chloramine T method, such as that of Prockop and Udenfriend (1960), where hydroxyproline is oxidised in the presence of a measured excess of alanine. Alternatively, the

other amino-acids may be separated by ion-exchange column chromatography together with many other interfering substances and this was the method used in this study.

Column chromatography using an ion-exchange resin was first described by Stein and Moore (1950). They used a sulphonated polystyrene resin column to elute the amino-acids in cationic form with increasing concentrations of hydrochloric acid. To achieve the more complete separation and differentiation they recommended eluting the column with a series of citrate buffers in a pH range from 3 to 7 (Moore and Stein, 1951). In this technique the ion-exchange resin, 8% cross-linked Dowex 50-H^+ was used in two columns; one of 100 cm height for acidic and neutral amino-acids and a second of 15 cm for the basic ones.

As only proline and hydroxyproline required separation for this study, it was sufficient to use a short 10 cm resin column eluted with 2N hydrochloric acid (Stern et al, 1963). These workers had used a Dowex 50-H^+ X-12 resin with 13-23 particle size, and found that hydroxyproline was eluted in the 8-16 ml fraction and proline between 20 and 28 ml. It was decided to use a similar resin in this study and to collect fractions to be analysed colorimetrically for hydroxyproline by the method of Bergman and Loxley (1963). The radioactivity of H^3-proline and H^3-hydroxyproline in aliquots from the fractions was determined in an automatic liquid scintillation counter.

Tritium autoradiography in organ culture.

Autoradiography is a technique for the detection and quantitation of radioactive isotopes by visualisation on a

photographic emulsion. By placing this in contact with histological sections from tissues pre-labelled with the isotope its distribution within cells and organic matrix can be accurately determined.

Autoradiography has been used to identify the sites of active bone and cartilage formation both in the intact animal and in vitro culture. Many isotopes can be used for this purpose. The formation of sulphated acid mucopolysaccharide in the cartilage matrix can be traced by the incorporation of sulphur 35 labelled compounds as demonstrated in vivo by Dziewiatkowski (1951) and by Fell et al (1956) in chick limb bone rudiments grown in tissue culture. Calcium 45 may be used in vivo (Owen, 1956) to identify the site of new bone formation but depends on normal calcification of the osteoid matrix which may produce difficulties in histological processing.

Either tritium (H^3) or carbon 14 labelled amino-acids may be utilised to show their incorporation into newly formed matrix protein. Carneiro and Leblond (1959) used an H^3-glycine label in mice because of its high content in collagen and Tonna (1961) extended this to the use of H^3-proline which is specifically located in the collagen molecule.

The first in vitro localisation of a labelled amino-acid was demonstrated by Gaillard (1961) who incorporated C^{14} glycine in his culture medium and demonstrated heavy blackening over the osteoblastic areas in the matrix of the mouse radius shaft. Later Heersche and Voogd Van Der Straaten (1965) used an H^3-proline label with the same model and showed even heavier labelling of

the osteoid matrix beneath the deep layers of the periosteum in the bony shaft. Lewis and Irving (1970) also demonstrated H^3-proline label incorporation into the bone matrix of rat calvaria in culture. Using embryonic chick bone rudiments, Ramp and Neuman (1971) showed some H^3-proline label was incorporated in matrix of the cartilaginous ends, though less than in the bone of the shaft.

In these experiments an H^3-proline label was added to the synthetic BGJ culture medium, in a concentration of 1.0 Ci/ml to identify the sites of collagen synthesis by autoradiography in parallel with the biochemical studies.

Collagen metabolism in hyperoxia.

Several workers have used organ culture of skeletal tissues to investigate the synthesis and resorption of collagen under conditions of hyperoxia by the techniques outlined above.

Stern et al (1963) studied the active resorption of bone collagen from six-day-old mouse calvaria, pre-labelled with H^3-proline in utero, induced by 50% oxygen tension with and without the addition of parathyroid extract. Their findings indicated that the collagen was enzymatically degraded during the oxygen induced bone resorption, but gave no indication of the effect on synthesis. In later experiments (Stern et al, 1966) these workers incorporated a known dose of H^3-proline in the natural media used for culture of the calvaria under similar conditions. This enabled them to use the incorporation of H^3-proline from the medium into collagen H^3-hydroxyproline as an index of collagen synthesis, and the release of hydroxyproline

into the medium as an index of collagen degradation. They showed that at low oxygen tensions the rate of collagen synthesis exceeds that of degradation but at higher oxygen tensions both the rates of synthesis and degradation increase.

Asher and Sledge (1968) used a similar H^3-labelled proline technique to investigate the collagen turnover in 12-day-old chick embryo mandibles exposed to 80% oxygen. Compared with controls grown in air, there was a 12% increase in resorption of collagen formed during the culture period, but no increase in the resorption of old pre-explantation collagen.

No previous studies have been made on collagen metabolism in mammalian limb bone rudiments exposed to hyperoxia. These experiments were carried out to complement the histological studies reported in Part III of this thesis. Answers were sought to the questions:-

(1) Is collagen synthesised in the mammalian limb bone rudiment during in vitro culture?

(2) Is this synthesis increased or decreased by hyperoxia?

(3) Is the degradation of collagen in the rudiments increased by hyperoxia?

(4) Is there more resorption of old pre-explantation or newly synthesised collagen?

(5) Is the collagen synthesis and degradation greater in the bone or the cartilage of the skeletal rudiments?

MATERIALS AND METHODS

1. Mice

Pregnant female mice of 17 - 19 days' gestation were used in these experiments to provide intermediate or late foetal limb bone rudiments with the maximum quantity of tissue for biochemical analysis.

2. Isolation of Bone Rudiments

Paired radii, ulnae and tibiae were dissected from the upper and lower limbs of the foetal mice in Tyrode's balanced salt solution using the aseptic technique described in Part I.

3. Culture Technique

The technique of culture was that described in Part I, using a metal grid to support the rudiments at the interphase between medium and gas. To produce a detectable level of hydroxyproline release into the media the number of rudiments on each grid was increased to twelve. The volume of culture medium in each dish was 1.5 ml. Three gaseous environments were used: 95% air with 5% carbon dioxide, 95% oxygen with 5% carbon dioxide, and 98% oxygen with 2% carbon dioxide at two atmospheres absolute pressure. Because of the risk of radioactive contamination between experiments, all dishes and grids were discarded at the completion of each culture. At the time of explantation extra pairs of rudiments were removed from the litter mates of the mice used for the experiment, to provide zero controls.

4. Media

The P6 modification of BGJ medium, containing no chemical proline or hydroxyproline, was used in all these experiments. Prior to final sterile filtration into 1.5 ml. aliquots this was labelled by the addition of tritium labelled proline. This was used in a concentration of 0.1 µci/ml. for the biochemical experiments, or 1.0 µci/ml. for the autoradiography studies. L-proline-5-H3 (batch no. TRA/K.323) was supplied by the Radiochemical Centre, Amersham with a specific activity of 6.8 Ci/mM. The amino-acid was supplied as a sterile aqueous solution in a rubber capped vial with a radioactive concentration of 0.5 mCi per ml. and a radiochemical purity over 98% by paper chromatography. Suitable dilutions were made with sterile double distilled water prior to adding the label to the culture medium.

5. Sub-Culture

Rudiments were cultured for six days in vitro; the media were changed every 48 hours and the cultures regassed after every 24 hours, using the technique described in Part I. Media were pooled for each set of experiments with the same gas phase and stored frozen until required for analysis. The rudiments harvested from each experiment were stored in their final medium at the completion of culture.

6. Histological Controls

a) Culture controls.

In each biochemical experiment a separate dish was

used to culture one set of paired rudiments under identical conditions. These were fixed, processed and stained by the routine histological methods described in Part I, to act as a control on culture conditions. Any rudiments failing to elongate or showing any evidence of infection were discarded, together with the remainder of the biochemical culture.

b) Autoradiography.

In three pairs of experiments comparing different gas phases, the histology controls were grown on media containing 1.0 µci/ml. of tritiated proline to give improved label incorporation for autoradiography. These rudiments were processed as described in the Materials and Methods section of Part I and after development were stained with toluidine blue or Harris haematoxylin. From these slides it was hoped to determine the histological localisation of the isotope and to identify the site of new collagen formation.

7. Biochemical Analysis

a) Rudiments - wet and dry weights.

After thawing, rudiments were washed three times in Tyrode's balanced salt solution to remove any residual media radioactivity, and blotted dry. They were placed in screw-topped pre-weighed polycarbonate centrifuge tubes (Sterilin Ltd.), which were then reweighed to determine the wet weight of the tissue. Rudiments

from three experiments were pooled to give thirty-six in each tube, or eighteen in the case of the zero controls. The tubes were placed in an oven and dried at 105°C for twelve hours and then reweighed to determine the dry weight of tissue.

b) Hydrolysis.

Prior to hydrolysis, a measured aliquot of 12 ml. from the 13.5 ml. of pooled medium was lyophilised in a Buchler evaporator using an alcohol/dry ice mixture. The freeze-dried precipitate was resuspended in 4 ml. of 6N hydrochloric acid and this was transferred to screw-topped polycarbonate tubes. A similar volume of 4 ml. of 6N hydrochloric acid was also added to each of the tubes containing rudiments. Rudiments and media were then hydrolysed at 105°C for twenty-four hours. After filtration the hydrolysates were evaporated to dryness and resuspended in 2 ml. of distilled water, from which a 1 ml. aliquot was taken for column chromatography.

c) Column Chromatography.

Glass columns were prepared containing 0.9 cm. x 10.0 cm. of Dowex 50-H+, X-12 resin of 200-400 mesh size (Sigma Chemicals Ltd.). These were equilibrated and eluted with 2N hydrochloric acid at room temperature using gravity flow from a reservoir at a rate of 1 ml. per minute. Using known standard

solutions of the amino-acids it was established that hydroxyproline came off in the fraction from 6 to 14 ml. and the proline from 16 - 28 ml. When each experimental sample was placed on the column and eluted these appropriate fractions were collected for analysis. Between runs the columns were regenerated with 2N sodium hydroxide to remove the basic amino-acids before reacidification.

d) Colorimetric Estimation for Hydroxyproline. (Method - Appendix C).

Duplicate 0.5 ml. samples were taken into graduated test tubes from the chromatographic fractions to be analysed for chemical hydroxyproline content. Using phenolphthalein as an indicator sufficient drops of 4N sodium hydroxide were added to produce a red colour. Drops of 4N hydrochloric acid were added by pipette until the alkali was neutralised and the solution became colourless. A similar volume of 4N sodium chloride was added to the standard solution of hydroxyproline used for comparison, to keep the salt concentration similar to that in the unknowns. All tubes were made up to a volume of 1.5 ml. with distilled water and then 1.0 ml. of isopropanol and 0.5 ml. of chloramine T solution were added. After standing for four-and-a-half minutes a 6.5 ml. volume

of Ehrlich's solution was added to each of the tubes, which were covered and allowed to stand overnight at room temperature. The colours of the solutions were then read against distilled water blanks in a Pye Unicam SP II spectrophotometer at an absorbence of 558 mμ. The corresponding hydroxyproline value was read off from a calibration curve, previously prepared from known hydroxyproline standards.

e) Radioactive Assay.

Duplicate 1.0 ml. samples from the hydroxyproline and proline fractions were pipetted into counting vials for determination of their radioactivity. This was compared with 0.5 ml. of the uncultured labelled medium added to 0.5 ml. of 2N hydrochloric acid as a standard. After the addition of 3 ml. of Triton-X and 6 ml. of NE 233 scintillator to each vial the samples were counted in an automatic liquid scintillation counter. The percentage distribution of labelled hydroxyproline between rudiments and media could then be calculated.

8. Design of Experiments

The maximum number of paired rudiments which could be handled conveniently in one experiment was twelve. These were cultured for a six-day period in two different gas phases to compare the synthesis and resorption of collagen in otherwise matched tissue. Six paired rudiments taken from the same litter were analysed for their hydroxyproline content to act as an approximate zero control for the

experiment. To obtain sufficient material for biochemical analysis the rudiments and media from three experiments using the same two gas phases were pooled. Nine experiments were carried out for each set of gas comparisons giving a mean of three sets of results.

Thus collagen synthesis and resorption were compared in paired rudiments grown under:-

a) 95% air + 5% CO_2 or 98% O_2 + 2% CO_2 at two atmospheres absolute pressure.

b) 95% O_2 + 5% CO_2 or 98% O_2 + 2% CO_2 at two atmospheres absolute pressure.

RESULTS

1) Biochemical estimation of Hydroxyproline.

a) Comparison of Air and Hyperbaric Oxygen (HBO).

Table 8 summarises the results from three sets of experiments estimating the biochemical content of hydroxyproline in rudiments and media after 6 days in culture. In both gas phases there was a synthesis of hydroxyproline compared with control rudiments taken at the time of explantation. The total of hydroxyproline synthesized was greatest in air and was double that in hyperbaric oxygen. The quantity of hydroxyproline released into the medium was also large and in hyperbaric oxygen was equal to, or exceeded, that synthesized during culture.

b) Comparison of 95% Oxygen and Hyperbaric Oxygen (HBO).

The results of three sets of experiments comparing these gas phases are shown in Table 9. The total of hydroxyproline synthesized was low in both types of hyperoxia, though it was certainly greater at hyperbaric pressure. Release of hydroxyproline into the medium was correspondingly high with both gas phases and resulted in a negative balance in the rudiments in half the experiments, with both normobaric and hyperbaric oxygen.

2) Radiochemical estimation of new ^{3}H - hydroxyproline partition.

The radiochemical results from all six experiments using the three gas phases are summarised in Table 10. The percentage of newly synthesized ^{3}H-hydroxyproline released into the medium was very high in all the experiments after the six days in culture. This resorption was almost equal in air and normobaric oxygen at 77 - 78%, but was somewhat reduced to 65% in hyperbaric oxygen

TABLE 8

HYDROXYPROLINE (OHP) CONTENT OF EXPLANTS AND MEDIA AFTER 6 DAYS IN VITRO.

Experiment	Wet Wt. (mgm)	Dry Wt. (mgm)	OHP RECOVERED (μgm)			OHP SYNTHESIZED (μgm)		
			Explants	Medium	Total	Total	Explants	Medium
1. Controls	63.2	7.2	188.10	-	-	-	-	-
Air	72.4	12.3	232.07	217.43	449.40	261.40	43.97	217.43
HBO	56.6	8.9	188.10	91.18	279.28	91.18	0	91.18
2. Controls	39.6	5.4	84.58	-	-	-	-	-
Air	43.1	7.3	127.72	151.51	279.23	194.65	43.14	151.51
HBO	34.4	5.5	102.40	62.61	165.01	80.43	17.82	62.61
3. Controls	53.4	7.8	99.66	-	-	-	-	-
Air	83.3	13.5	165.42	88.89	254.31	154.65	65.76	88.89
HBO	51.4	9.1	96.07	77.55	173.62	73.96	-3.59	77.55

MEAN OHP SYNTHESIS - IN AIR 203.6 μgm $\pm$ 53.9

- IN HYPERBARIC OXYGEN 81.8 μgm $\pm$ 8.7

TABLE 9

HYDROXYPROLINE (OHP) CONTENT OF EXPLANTS AND MEDIA AFTER 6 DAYS IN VITRO.

Experiment	Wet Wt. (mgm)	Dry Wt. (mgm)	OHP RECOVERED (μgm)			OHP SYNTHESIZED (μgm)		
			Explants	Medium	Total	Total	Explants	Medium
4. Controls		6.2	54.66	-	-	-	-	-
Oxygen		5.7	46.97	41.68	88.65	33.99	-7.69	41.68
HBO		5.7	58.65	52.33	110.98	56.32	3.99	52.33
5. Controls	50.6	9.4	99.96	-	-	-	-	-
Oxygen	47.8	7.7	99.68	39.74	139.42	39.46	-0.28	39.74
HBO	44.4	7.4	94.51	70.44	164.95	64.99	-5.45	70.44
6. Controls	47.2	6.2	116.04	-	-	-	-	-
Oxygen	51.1	8.1	118.77	28.27	147.04	31.00	2.73	28.27
HBO	48.1	7.9	121.52	51.82	173.34	57.30	5.48	51.82

MEAN OHP SYNTHESIS - IN OXYGEN 34.8 μgm ± 4.3

- IN HYPERBARIC OXYGEN 59.5 μgm ± 4.7

TABLE 10

SYNTHESIS AND RELEASE OF ^{3}H-HYDROXYPROLINE AFTER 6 DAYS IN VITRO.

Experiment	^{3}H-Hydroxyproline Recovered (CPM)			% of Total ^{3}H-Hydroxyproline Recovered	
	Explants	Medium	Total	Explant	Medium
1. Air	34,096	302,532	336,628	10.1%	89.9%
HBO	7,080	3,939	11,019	64.2%	35.8%
2. Air	17,688	31,104	48,792	36.3%	63.7%
HBO	4,568	6,696	11,264	40.5%	59.5%
3. Air	25,790	97,500	123,290	20.9%	79.1%
HBO	13,420	31,974	45,394	29.6%	70.4%
4. Oxygen	1,768	7,144	8,912	19.8%	80.2%
HBO	2,352	5,832	8,184	28.7%	71.3%
5. Oxygen	1,226	8,483	9,709	12.6%	87.4%
HBO	1,240	6,975	8,215	15.1%	84.9%
6. Oxygen	3,584	7,303	10,887	32.9%	67.1%
HBO	3,224	6,480	9,704	33.2%	66.8%

MEAN % OF ^{3}H-HYDROXYPROLINE RELEASED IN MEDIUM

- IN AIR 77.6% $\pm$ 13.2
- IN OXYGEN 78.2% $\pm$ 10.3
- IN HYPERBARIC OXYGEN 64.8% $\pm$ 16.4

with more labelled hydroxyproline remaining in the rudiments.

3) Pre-explantation Hydroxyproline resorbed during culture.

The amount of 'old' pre-explantation hydroxyproline resorbed and released into the medium was calculated in each experiment and is shown in Table 11. In air resorption of this 'old' hydroxyproline only occurred in one of the three experiments, but was a constant feature with both conditions of hyperoxia. The greatest release of pre-explantation collagen occurred with hyperbaric oxygen which contrasts with the smaller release of newly synthesized hydroxyproline shown in Table 10.

In summary, the biochemical and radiochemical results showed a net increase of resorption over synthesis with both types of hyperoxia. In air the synthesis of 'new' labelled hydroxyproline was much greater, but nearly 80% of this was released again into the medium, in preference to the 'old' pre-explantation hydroxyproline.

TABLE 11

"OLD" HYDROXYPROLINE RESORBED DURING CULTURE PERIOD.

Experiment	MEDIA OHP (µgm) (M_1)	TOTAL OHP SYNTHESIZED (µgm) (T)	% LABELLED OHP IN MEDIUM ($^3_{HM}$)	QUANTITY OF NEWLY SYNTHESIZED OHP RESORBED (µgm) (M_2)	PRE-EXPLANTATION "OLD" OHP RESORBED DURING CULTURE PERIOD (µgm) ($M_1 - M_2$)
1. Air	217.4	261.4	89.9	234.9	O
HBO	91.2	91.2	35.8	32.6	58.6
2. Air	151.5	194.6	63.7	123.9	28.6
HBO	62.6	80.4	59.5	47.8	14.8
3. Air	88.9	154.6	79.1	122.3	O
HBO	77.5	73.9	70.4	52.1	25.4
4. Oxygen	41.7	33.9	80.2	27.2	14.4
HBO	52.3	56.3	71.3	40.2	12.1
5. Oxygen	39.7	39.4	87.4	34.5	5.2
HBO	70.4	64.9	84.9	55.2	15.2
6. Oxygen	28.3	31.0	67.1	20.8	7.4
HBO	51.8	57.3	66.8	38.3	13.5

N.B. $M_2 = \frac{T \times {}^3_{HM}}{100}$

DISCUSSION

1) Synthesis of collagen in mammalian limb bones in vitro.

The results of autoradiography reported in Parts I and III showing incorporation of tritiated proline label into bone and cartilage suggested that collagen could be formed in these rudiments in culture. The possibility remained that the labelled proline was incorporated in some collagen precursor, such as protocollagen, and that further conversion then ceased. The biochemical and radiochemical results reported here confirmed that ^{3}H-hydroxyproline was synthesised and appeared in the rudiments and media. Since the only significant tissue protein containing hydroxyproline was shown by Prockop and Kivirikko (1967) to be collagen its formation seems proven.

No comparable figures are available for mammalian limb bone rudiments in vitro, though Halme et al (1969) studied the incorporation of ^{14}C-proline into the embryonic mouse ulna. They reported optimum conversion to ^{14}C-hydroxyproline occurred in air when the medium had a high ascorbate level and was changed daily. If these conditions were not observed during the six day period of culture, the proline incorporation fell to 60% and less was in the form of hydroxyproline. Absence of ascorbate, oxygen, ferrous iron, or α-ketoglutarate resulted in over half the labelled proline being in the form of protocollagen, a hydroxyproline deficient precursor. The importance of the stimulatory action of ascorbic acid on collagen synthesis was confirmed by Birge and Peck (1966)

using isolated bone cells from rat calvaria. The high level of ascorbate in the modified BGJ medium used in these experiments would appear adequate, although its level may fall in prolonged exposure to hyperoxia.

2) Effect of hyperoxia on collagen synthesis.

The overall effect of hyperoxia is to decrease the synthesis of collagen. From the results reported it appears that a little more is formed in hyperbaric oxygen, than in 95% oxygen at ambient pressure. From the histological findings reported in Part III it might be concluded that the difference reflected the diminished resorption in hyperbaric oxygen, but the biochemical results suggest that more collagen was in fact synthesised under these conditions. The mechanism is not clear but may be related to the initial stimulatory effect on periosteal osteoblasts seen with hyperbaric oxygen.

The results at ambient pressure conform with those obtained by Stern et al (1966) using mouse calvaria in vitro. They showed an increased incorporation of ^{3}H-hydroxyproline with increasing oxygen concentration up to a maximum at 30%, but at higher levels the rate of resorption also increased. Chvapil and Hurych (1968) reviewed the evidence that the oxygen tension controls all the steps in the metabolism of collagenous proteins, but has little effect on non-collagenous ones. They showed that proline hydroxylation was deficient when the oxygen concentration was less than 10%, but that over 20% no real increase in synthesis occurred, although collagen resorption was stimulated.

3) Effect of hyperoxia on collagen degradation.

The biochemical results confirmed the histological findings in Part III that hyperoxia increased the degradation of collagen. Like synthesis, the effects at hyperbaric and ambient pressures were different and would fit the greater resorption by osteoclasts and osteocytic osteolysis observed in 95% oxygen.

4) Resorption of 'old' and 'new' collagen.

The results in these experiments suggest that the majority of the collagen resorbed and degraded in the rudiments was that newly formed during the period of culture, with only a small quantity from pre-explantation sources. There is some controversy in the literature as to which type is most susceptible. Stern et al (1966) reported that more 'old' collagen was resorbed in calvaria exposed to 50% oxygen and that a constant fraction of 20 - 30% of the 'new' collagen not maturing was released into the medium. They attributed the increased resorption of the 'old' collagen to its incorporation in calcified matrix which was more susceptible to osteoclast activity. This might explain the different results reported here, with a mixed rudiment containing relatively less calcified matrix and where osteocytosis was as active as osteoclasia in resorption. They accord more with the findings of Asher and Sledge (1968) using chick mandibles, who reported that 95% oxygen increased the resorption of newly formed collagen from the 12% in air to 80%, but had no effect on pre-explantation collagen. Prockop and Kivirikko (1967)

used in vivo isotope studies to show that one-third to one-half of the hydroxyproline excreted in young animals originates from the degradation of newly formed 'soluble' collagens. With increasing age more hydroxyproline originates from the metabolically stable 'insoluble' collagen. As embryonic rudiments were used in the experiments reported here, the former situation would be expected to obtain. Prockop and Kivirikko also showed that once released the hydroxyproline is not normally re-incorporated into newly synthesised collagen, but that free proline is rapidly incorporated. This fact simplifies the interpretation of the biochemical and radiochemical data and was confirmed by the in vitro metabolic studies of Flanagan and Nichols (1968) using rat metaphyseal bone slices.

5) Comparison of collagen synthesis and degradation in bone and cartilage.

No biochemical studies were done on the relative synthesis and degradation of collagen in bone and cartilage, though this might form the basis of future work. The results of autoradiography would agree with Gaillard (1961) and Heersche and de Voogd van der Straaten (1965), who showed heaviest ^{3}H-amino acid label in the osteoid of the bony shaft deep to the active osteoblastic territories. Some light labelling occurred in the matrix of the end cartilages, particularly in the zone of proliferating chondrocytes, but this was never as marked as in bone. Similarly, resorption of label induced by hyperoxia appeared to be greatest in the bone with more preservation in the cartilage matrix. Interpretations from

these morphological data are open to question since it is not known whether the isotope label is in the form of proline in protocollagen or hydroxyproline in mature collagen.

PART V

THE ROLE OF LYSOSOMAL ENZYMES IN BONE AND CARTILAGE DEGRADATION

INTRODUCTION

Properties of lysosomal enzymes.

The intracellular cytoplasmic granules termed lysosomes, which contain acid hydrolase enzymes were first described by de Duve (1959) in the cells of liver and kidney. When their role in tissue breakdown was recognized they were studied in other cells, including those of the connective tissues. The evidence for their presence and role in cartilage and bone has been well reviewed by Vaes (1969) and the properties of their contained enzymes by Barrett (1969). Of the 30-40 lysosomal enzymes identified, those of most interest in skeletal physiology are acid phosphatase, beta-glucuronidase, and acid protease or cathepsin D. It appears that several iso-enzymes may occur, showing species differences in both their substrate activity and optimum pH.

Acid phosphatase from mammalian tissue preparations will hydrolyse a variety of phosphoric monoesters under acidic conditions, with an optimum pH of from 4 to 5.

Beta-glucuronidase can be found in both lysosomes and endoplasmic reticulum from mammalian cells. It removes terminal non-reducing residues of glucuronic acid from the oligosaccharides, which are produced when hyaluronidase acts on hyaluronic acid and chondroitin sulphate. It has an optimum pH between 4.3 and 5.2.

Acid proteases of several types are found in lysosomes but cathepsin D is the one of most interest in connective tissue catabolism. It has few low molecular weight substrates but will act on many proteins, in a manner similar to that of pepsin, with

an optimum pH of 3.0 to 3.5. It produces protein degradation with liberation of amino-acids by an imino transfer mechanism.

The lysosomal enzymes may be identified by both biochemical and histochemical methods. Biochemical estimations can be carried out on homogenates of both cells and tissues and also culture media used for in vitro growth. Using suitable enzyme substrates the enzymes can also be identified in intact tissue by histochemical staining.

Role in cartilage.

Fell & Mellanby (1952) described the effect of vitamin A excess on early cartilaginous limb bone rudiments from chick embryos grown in tissue culture. They obtained marked resorption of the cartilage with loss of metachromatic staining in the ground substance, but were unsure of the mechanism. Lucy et al (1961) demonstrated that an extract from the same tissue had strong proteolytic enzyme activity with an optimum pH of 3. The enzymes were located in cytoplasmic particles present in normal chondrocytes and could produce a loss of matrix staining similar to an excess of vitamin A. Dingle (1961) showed that particulate preparations from rat liver, now known to be lysosomes, released a similar proteolytic enzyme when treated with vitamin A. Finally Fell & Dingle (1963) demonstrated that vitamin A caused the release of the protease, both from cartilage rudiments in culture and the lysosomal particulate fraction prepared from them. This was capable of degrading ground substance and releasing chondroitin sulphate at a physiological pH.

Sledge and Dingle (1965) reported the production of

histological changes, similar to those from excess of vitamin A, in cartilaginous chick limb bone rudiments exposed to elevated partial pressures of oxygen. This was accompanied by an increased production and release of lysosomal acid phosphatase and acide protease. They postulated that the mechanism might occur physiologically in the growth plate, when the avascular cartilage is invaded by the metaphyseal blood vessels producing degradation of the ground substance and replacement by bone. In later studies, Sledge (1968) proposed a similar mechanism for the formation of a marrow cavity and demonstrated this near the upper surface of cartilaginous rudiments exposed to hyperoxia, when their lower surfaces were protected by a plasma clot. By histochemical staining he showed the sites of acid phosphatase activity within the chondrocytes in the epiphyseal plate from newborn mice and demonstrated that the activity was most intense in the hypertrophic and proliferative cell layers adjacent to the vessels. Kuhlman (1965) also reported high concentrations of acid phosphatase in these zones by direct microchemical analysis of the rabbit epiphyseal plate. The mucopolysaccharides of cartilage ground substance probably have a dual function of stabilizing collagen and preventing calcium phosphate precipitation, so that their breakdown by enzymes must precede calcification in the epiphyseal plate. Evidence for this was provided by Campo and Dziewiatkowski (1963) in 8-10 day old rats using a simultaneous radioactive label with S_{35} for chondroitin sulphate and C_{14} for protein. After the initial double labelling of the chondrocytes, they showed progressive changes leading to a complete loss of the C_{14} labelled

protein but persistence of the S_{35} labelled mucopolysaccharide in the cores of the metaphyseal bone spicules.

Woesnner (1967) was able to isolate and purify a lysosomal enzyme, cathepsin D, from cartilage which accounts for the major protein digesting activity at acid pH. Although the optimum pH for mucopolysaccharide digestion lies between 4.0 and 4.5 he showed significant digestion at pH 7.0.

The lysosomal enzyme degradation of the non-collagenous proteins of cartilage has been established, but the explanation for collagenolytic activity has been less satisfactory. Since the turnover of collagen in bone is amongst the highest in collagenous tissues the problem will be considered under that heading.

Role in bone.

Two main mechanisms are recognized for the resorption of bone matrix; the osteoclastic activity at resorption surfaces, which was well described by Hancox (1956), and the 'osteolysis' surrounding osteocytes which are activated by certain stimuli as outlined by Belanger (1963). Belanger (1961) has suggested that this mechanism may be as important as the better documented osteoclast resorption in bone. He described two types of osteocyte; one a small inactive cell in a smooth lacuna, and the other a larger cell shown histochemically to contain both alkaline phosphatase and acid protease. This type lies in a rough-edged, enlarging cavity prior to final death and lysis. The surrounding organic matrix was modified and partially removed reverting to the metachromatic staining characteristic of acid mucopolysaccharide after the release of bone salts.

The identification of lysosomal enzymes in bone was delayed by the difficulty of producing an homogenate of such hard tissue and the heterogenous cell population which resulted if long bones were used. To overcome this histochemical methods were used first to identify the sites of activity. Burstone (1960) was able to demonstrate acid phosphatase and aminopeptidase activity in the cytoplasm of phagocytic cells, including osteoclasts, although its function was unknown. Hancox and Boothroyd (1963) studied osteoclast function in embryonic avian bone by electron microscopy and demonstrated continuous matrix digestion at their 'ruffled' border. The bone crystals from the matrix were resorbed first, entering channels and vacuoles in the cytoplasm, and were then followed by the collagen fibrils. From comparison with the histochemical distribution of lysosomal enzymes, they suggested that these were responsible for the resorption. Belanger and Migicovsky (1963) were able to identify protease activity around the large osteocytes in young chick and rat bone by histochemical means.

Vaes and Jacques (1965) identified nine lysosomal enzymes by biochemical methods in homogenates from newborn rat calvaria, using this model to minimise contamination from marrow and cartilage cells. The enzymes were all most active at an acid pH and included acid phosphatase, cathepsin, and beta-glucuronidase, with similar or higher specific activities than those derived from liver. A correlation between increased histochemical and biochemical acid phosphatase activity, in mouse calvaria resorbing in culture under the stimulus of parathyroid hormone, was shown by

Susi et al (1966). The histochemical activity was most intense in the cytoplasm of the numerous osteoclasts associated with the areas of resorbing bone, though slight activity was also seen within the larger osteocytes.

Vaes (1968) using a similar calvaria model demonstrated the release of six acid hydrolase enzymes into the culture media and correlated this with the bone resorption as estimated by the release of bone mineral, calcium$_{45}$ label, hexosamine and hydroxyproline. He also showed that the intracellular enzyme stores were maintained by new synthesis and that these were released by a process of exocytosis and not lysosomal membrane disruption, with an associated acidification of the medium in the resorbing cultures. Vaes postulated that this acidification, possibly resulting from the stimulation of glycolysis, results in solubilisation of the bone mineral assisting the hydrolytic action of the enzymes.

Tolnai (1968) obtained similar results when she studied the biochemical release of acid protease, acid phosphatase, and beta-glucuronidase activity in the resorbing rat and mouse calvaria stimulated with parathyroid hormone. Although she showed an increased release of all three enzymes into the culture media, an increased synthesis only occurred with acid protease and phosphatase and not with beta-glucuronidase, suggesting a possible individual control of enzyme activity in the vacuolar system of bone cells.

Collagenases in bone and cartilage.

The major defect in the postulated role of the lysosomes in the degradation of bone and cartilage has been the persistent

failure to identify a collagenase among the acid hydrolase enzymes liberated from these bodies. Collagenolytic activity, capable of degrading collagen pre-labelled with tritiated proline and hydroxyproline, has been demonstrated in resorbing bone in culture by Kaufman et al (1965). They laid reconstituted labelled collagen fibrils on mouse calvaria and stimulated resorption with parathyroid hormone and heparin. Nagai et al (1966) in attempting to prepare and purify a collagenase enzyme from the tissues of the tadpole tail showed that mammalian collagen in solution at an acid pH will undergo progressive denaturation at temperatures above 30°C. This raises the question as to whether collagen breakdown in bone and cartilage is initiated by an acid pH in the resorbing areas, permitting lysosomal enzymes to complete the proteolytic digestion. A collagenase enzyme was identified and characterised by Shimizu et al (1969) from five-day-old mouse tibiae in culture. It was found to have an optimum pH of 7.0 to 8.6, but no activity at an acid pH of 5.0. This fact, combined with the inability to extract the enzyme from tissue homogenates treated to disrupt their lysosomal membranes, supports the concept that collagenase enzyme is not of lysosomal origin. Contrary evidence was provided by the finding of Wood and Nichols (1965), that a collagenase extracted from an homogenate of rat bone cells had an optimum pH of 6.0 suggesting a lysosomal origin. This discrepancy could arise if the lysosomal collagenase were to show latent activity, as suggested by Verity et al (1968) as a property conferred by a differing enzyme-membrane bond. Another alternative is that the enzyme is never stored inside cells, but is excreted as

soon as it is synthesised, which might be necessary to preserve the integrity of the cell itself.

Inhibition of bone and cartilage resorption.

If lysosomal enzymes are responsible for bone and cartilage resorption it would be expected that substances capable of blocking their release or inhibiting their activity would prevent this process occurring. Such agents might react directly with the stimulus to lysosomal activation, they might prevent its entry to the cells, or combine with its receptor sites. Alternatively they might stabilize the substrate, interfere with the cellular action or differentiation, or with some metabolic step in the synthesis of the enzymes themselves.

Action of hydrocortisone and cortisone on bone and cartilage.

Hydrocortisone is thought to stabilize lysosomal membranes and prevent the release of their contained enzymes. This effect was demonstrated by Weissman and Dingle (1961) who showed that pretreatment of rats with hydrocortisone inhibited the release of protease enzyme from their liver lysosomes when these were exposed to breakdown by ultra-violet irradiation.

The effect of hydrocortisone on cartilage in tissue culture has been studied by Buno and Goyena (1955) using an embryonic chick femur model. They showed that it inhibited the growth of the cartilaginous rudiment, probably by reducing cell division, but produced no difference in the matrix when compared with controls. Fell and Thomas (1961) confirmed these findings using a dosage of 7.5 g/ml of medium and reported thinning of the intercellular matrix partitions, though often with more intense

metachromatic staining in the ground substance, and smaller diaphyseal cells. They suggested that the hormone produced a combined effect of depressed matrix synthesis with a failure of hypertrophy in the diaphyseal chondroblasts. By contrast, in the mixed cartilage and bone model of the late foetal mouse limb bone, hydrocortisone produced inhibition of cartilage resorption by the cells of the periosteum and endochondral regions. The same authors showed that exposure of both chick and mouse limb bone rudiments to high doses of Vitamin A resulted in resorption and disintegration, but that the effect of hydrocortisone was to retard, though not suppress this. This contrasts with the action of Vitamin A and hydrocortisone in vivo which appears to be additive, as Selye (1958) reported an increased bone resorption in the rat treated with both substances when compared with Vitamin A alone.

This led other workers to use hydrocortisone to inhibit the resorption produced in culture by other agents capable of liberating lysosomal enzymes. Sledge and Dingle (1965) showed that 0.1 g/ml of medium prevented the loss of matrix metachromasia and increased release of protease enzymes produced in embryonic chick cartilage by exposure to hyperoxia. Similarly the resorption of foetal mouse limb bones induced by antiserum with complement was prevented by 1.0 g/ml of hydrocortisone (Fell and Weiss, 1965). They reported that protease release was inhibited, but that immune cytolysis still occurred.

Goldhaber (1965) was unable to prevent bone resorption in foetal mouse calvaria exposed to Vitamin A or parathyroid hormone

using hydrocortisone in dosage from 10-100 g/ml. Slight inhibition of very low doses of Vitamin A and parathyroid hormone could only be obtained with massive doses of hydrocortisone, which in themselves produce toxic effects by inhibiting fibroblastic outgrowth. Surprisingly, a concentration of 10 g/ml enhanced the resorption produced by low doses of parathyroid hormone. This failure to prevent the calvaria resorption induced by Vitamin A was confirmed by Reynolds (1968), who also reported a definite inhibition of lysosomal protease release. This suggested that some mechanism, other than lysosomal enzymes, was responsible for bone resorption under these conditions.

The direct effects of cortico-steroids on bone and cartilage in tissue culture has also been studied. Schryver (1965), using low hydrocortisone concentrations of 1.0 g/ml and less, showed it inhibited growth in length and wet weight of embryonic chick cartilage, but did not decrease its dry weight or chondroitin sulphate synthesis. With even smaller doses of 0.01 g/ml, Reynolds (1966) reported a reduction in the hydration of the epiphyses, which normally occurs in chick bone rudiments grown on synthetic culture media. This was accompanied by an increase in collagen content, but a progressive decrease in polysaccharide and DNA synthesis. The dense metachromatic staining in the cartilage matrix was preserved, but the diaphyseal cells were smaller than in the control rudiments. This increase in collagen and fall in polysaccharide content approximates more closely to the in vivo situation. Normally the lack of ossification in vitro leads to a higher proportion of hexosamine in the rudiment. The inhibition

of chondroitin sulphate synthesis in chick cartilage was confirmed by Murota et al (1967), who demonstrated that hydrocortisone, but not its metabolites, decreased the incorporation of a S35 sulphate label into the ground substance.

These effects on cartilage in vitro accord well with the findings in vivo reported by Moscona and Karnofsky (1960). They reported that a single does of cortisone acetate administered to chick embryos retarded their skeletal development by inhibiting ossification in both endochondral and membrane bones. This interference with the conversion of cartilage to bone in endochondral ossification was confirmed by the autoradiographic study of Young and Crane (1964) in growing rats. Using a tritiated thymidine label they showed that hydrocortisone administered for 28 days caused narrowing of the epiphyseal cartilage with an associated reduction in the number of cells in the growth column, particularly the hypertrophied chondrocytes. There was a reduction in epiphyseal cartilage matrix formation as well as impaired cartilage resorption in the metaphyseal zone of provisional calcification. This failure of calcified cartilage resorption and its encasement in dense bone had previously been reported by Follis (1951) in the metaphysis of rats treated with cortisone for three weeks. This was thought to result from an inhibition of osteoclastic activity with continued normal osteoblast function.

In the more complex tissue of adult bone, cortico-steroids may interfere with other processes, such as calcium and phosphate metabolism and the release of bone mineral, masking its indirect action on the organic matrix. It may also accelerate the action

of other stimuli to bone resorption, such as the synergistic effect with Vitamin A described by Selye (1958).

In isolated bone cells in culture, Peck et al (1967) showed that hydrocortisone inhibits the incorporation of C14 proline into collagen and non-collagenous proteins. There was no associated increase in the rate of protein breakdown, but a 10% decrease in total tissue RNA content suggested the mechanism was an inhibition of RNA synthesis.

E-amino-caproic-acid (EACA)

Epsilon-amino-caproic-acid, or EACA, has been investigated experimentally as a possible inhibitor of bone and cartilage degradation, because of its probable action as an inhibitor of lysosomal protease enzymes. Ali (1964) showed that 0.4M EACA inhibited 40% of the proteolytic activity in cytoplasmic extracts of cartilage incubated in vitro to produce autolytic degradation at pH 5.0. The results were assessed by the release of chondro-mucoprotein and the loss of matrix metachromasia.

Sledge and Dingle (1965) reported that concentrations of 0.1M EACA largely prevented the effects of hyperoxia on embryonic chick cartilage, as shown by preservation of the matrix meta-chromatic staining and a reduction in protease release into the medium. At an even lower concentration of 0.05M, Fell et al (1966) showed that it inhibited the effect of complement and antiserum on 7½-day chick cartilaginous limb bone rudiments. The loss of muco-polysaccharide staining was prevented, but not the cell death from the immune reaction. Dingle et al (1967) used the same media dosage with 13-day chick limb bones exposed to the same stimulus

and demonstrated a 50% reduction in the release of hydroxyproline into the medium.

Evidence that the EACA acts directly on the enzymes was provided by Woessner (1967), who showed that the purified enzyme, cathepsin D, was partially inhibited by 0.1M EACA.

The effect of this inhibitor on mammalian bone resorption was reported by Brighton and Schaffzin (1970). Using 0.1M EACA they partially reversed the effects of 90% oxygen on the rat epiphyseal plate. This normally resulted in a loss of metachromatic staining in the cartilage ground substance with an increased secretion of PAS positive glycoprotein material at the cartilage-bone junction. The zone of calcified cartilage at the junction with the bony diaphysis was also preserved, instead of being narrowed or resorbed.

The effects of EACA on bone alone in culture were investigated by Vaes (1969). He reported that 0.1M EACA could partially inhibit the resorption of calvaria produced by parathyroid hormone, while this was totally abolished by a concentration of 0.2M.

Other known inhibitors of lysosomal enzymes, such as chloroquine, Vitamin E, and oestradiol, which might inhibit bone degradation have not been investigated in this study.

Investigation of the activity of lysosomal enzymes in hyperoxia.

The experiments reported in Part V of this thesis were designed to investigate the role of lysosomal enzymes in the changes induced in mammalian limb bone rudiments by hyperoxia. Answers were sought to the following questions:-

(1) Is there an increased production or release of

lysosomal enzymes in hyperoxia?

(2) Are all lysosomal enzymes equally affected, or is there a difference in the behaviour of individual enzymes?

(3) Can hydrocortisone, in a dosage of 1.0 or 5.0 gm/ml, inhibit the release of enzymes from lysosomes and what are the associated histological changes in the rudiments?

(4) Will EACA in a concentration of 0.1M block the action of lysosomal enzymes in hyperoxia?

MATERIALS AND METHODS

(1) Preparation of the explants.

Late 18-19 day foetal mouse limb bone rudiments were used for all the experiments in this part of the study. To obtain sufficient material for the lysosomal enzyme estimations it was necessary to use both fore and hind-limb rudiments from all the mice in each litter. Tibiae, radii and ulnae were prepared under sterile conditions using the technique described in Part I.

(2) Culture technique.

After preparation the paired rudiments were placed in separate small culture dishes supported on stainless steel grids at the medium-gas interphase, as described in Part I. Twelve rudiments were used on each grid with 1.5 ml of medium to obtain measurable levels of enzyme activity.

The medium was changed after each 48 hour period and the cultures regassed every 24 hours as described in Part I. After collection the medium was stored in the deep freeze together with the rudiments, which were harvested at the end of six days in culture. The media and rudiments were only thawed when required for enzyme estimations.

(3) Culture medium.

In all the experiments reported in Part V a modified BGJ medium (P6) developed by the Strangeways Laboratory, Cambridge was used. The commercial TC 199 medium contains phenol red as an indicator making it unsuitable for colorimetric reactions. The complete BGJ medium was supplied in

powdered form and was dissolved in double glass distilled water using the sterile filtration technique described in Part I. A supplement of 5% heat inactivated foetal calf serum (Burroughs Wellcome) and any enzyme inhibitor were added prior to final millipore filtration and dispensing in 1.5 ml aliquots, which were stored frozen until required for use.

(a) Hydrocortisone.

In experiments to test the ability of steroids to stabilise lysosomal enzymes, a medium was used containing hydrocortisone in a dosage of 1.0μgm/ml or 5.0μgm/ml. Hydrocortisone sodium succinate (Upjohn Ltd.) in powder form, equivalent to 100 mgm of hydrocortisone, was dissolved in 2 ml of water for injection. A 1 ml aliquot was removed and diluted with glass distilled water so that 0.1 ml of the resulting solution when added to 100 ml of bulk BGJ medium gave the necessary concentrations of 1.0μgm/ml or 5.0μgm/ml, prior to final filtration.

(b) E-amino-caproic acid.

EACA in powdered form (British Drug Houses Ltd.) was dissolved in sterile glass distilled water in a concentration of 60 mgm/ml. 7 ml of this solution was added to 100 ml of bulk BGJ medium prior to millipore filtration to give a final concentration of 40μgm/ml (0.1M).

(4) Methods of morphological examination.

(a) Growth in length.

In experiments to investigate the morphological effects of lysosomal enzyme inhibitors and blocking agents the growth in length of rudiments was determined. Rudiments were measured using the graticule eyepiece of the dissecting microscope at the time of explantation and at each medium change.

(b) Histological processing.

Rudiments for morphological studies were fixed, embedded, cut, and stained using the methods described in Part I of this thesis.

(5) Estimation of lysosomal enzymes.

(a) Preparation of rudiments.

(i) Homogenization - For biochemical estimation of enzyme activity in skeletal tissue or cells it is necessary to homogenise the tissue. This was done as gently as possible in order to minimise physical damage to the lysosomes and was scientifically the least well controlled part of the biochemical analysis. The suspending medium normally used was 1.5 ml of 0.25M sucrose which provides sufficient osmotic potential in the medium so that the particles did not swell or burst and liberate their enzyme content.

Mechanical cell breakage was normally achieved by use of an all glass homogenizer with a rotating

pestle. Mechanical instruments were not available so when this was used by hand the time and frequency of rotation were kept as constant as possible. It was important that this and all other techniques in the analysis were carried out at a temperature of 4-5°C in order to minimise the inactivation of these heat-labile enzymes.

(ii) Isolation of lysosomes - The only available physical method of isolating lysosomes from tissue homogenates was by differential centrifugation. Cellular debris were removed by preliminary spinning at 3,300 g for ten minutes. The light mitochondrial fraction, containing most of the lysosomes, was then sedimented by centrifugation at 10,000 g for 20 minutes. The supernatent was then assayed for "free" enzyme activity. The remaining "bound" enzyme activity was liberated from the lysosomal pellet by resuspending this in 1.5 ml of Triton X-100. Wattiaux and de Duve (1956) showed that this detergent was capable of releasing all bound forms of lysosomal enzymes by its ability to dissolve the lipid bound membrane. The critical detergent concentration causing abrupt release of the enzymes was 0.035% and the detergent was shown to have no inhibitory effect on the enzymes themselves.

(b) Determination of enzyme activity.

The extracts from the tissue homogenates and the culture media were assayed for activity of one or more

lysosomal enzymes using the following methods:-

(i) Acid phosphatase - Activity was determined biochemically using a modification of the method of Torriani (1960). This assay uses the ability of the enzyme to split off inorganic phosphate from a colourless nitrophenol-phosphate compound liberating nitrophenol which can be estimated colorimetrically. The reaction was carried out in an acid citrate buffer at a pH of 4.8. The incubation was carried out for 30 minutes at 37°C and then stopped by the addition of an excess of 0.1N sodium hydroxide giving a stable colour which was read in a spectrophotometer (Pye Unicam SP600) set to zero with the reagent blank at an optical density of 410. The activity of the enzyme corresponding to the reading was determined from a previously prepared calibration chart using known dilutions of a nitrophenol standard.

(ii) β-glucuronidase - was estimated biochemically by its action on a substrate of phenolphthalein glucuronidate (Fishman, 1967) with the liberation of phenolphthalein which was estimated colorimetrically. The reaction was carried out in an acetate buffer at a pH of 4.5 for 60 minutes at 37°C. At the end of incubation an excess of glycine buffer of pH 10.4 was added to stop the reaction. The optical density was read at 540 mμ in the spectrophotometer using a reagent blank as a zero reference. From previous standard calibrations using dilutions of a standard solution of phenolphthalein the amount of enzyme activity corresponding to the colour present in the reaction solution was determined.

(iii) Cathepsin D - was estimated by its protease activity on acid denatured haemoglobin solution. The method is based on that of Anson (1938) as modified to a micro method by Barrett (1967). Only 0.25 ml of 4% haemoglobin solution was required as substrate for 0.5 ml of the enzyme sample and incubation was carried out in a sodium formate buffer at a pH of 3.0 for one hour at 45°C. The reaction was then stopped by the addition of 5 ml of 3% trichloroacetic acid precipitating all the undigested protein from the solution. After filtration through Whatman No. 3 paper the liberated amino-acids could be read directly in the supernatent at an extinction value of 280 mμ if an ultraviolet spectrophotometer were available. In the absence of this instrument an alternative was to use a colorimetric reaction in the normal visual light wavelengths. An aliquot was placed in a clean test tube to which was added freshly prepared modified alkaline copper reagent and diluted Folin-Ciocalteau phenol reagent with immediate mixing. After standing for 30 minutes the blue colour was read against reagent blanks at an extinction value of 500 mμ. The colour reaction was standardised against tyrosine (up to 20 μg/tube) in 3% trichloroacetic acid.

RESULTS

1) Estimation of lysosomal enzymes.

a) Acid phosphatase.

Table 12 shows the results from two experiments, each using 48 paired rudiments, for comparison of three gas phases. The sequential levels of free enzyme activity in the media showed that the level in oxygen was slightly elevated at two days over the controls in air. This also occurred to a lesser extent with hyperbaric oxygen, but all media levels were equal at four days and surprisingly the highest levels were obtained in air after six days. The total activities in the media for the six day period of culture were highest in air and lowest with hyperbaric oxygen. Similarly the total of free and bound enzyme activity in the rudiments was highest in air, but lowest in oxygen suggesting that more enzyme was liberated in the medium. The percentage of free activity in the medium was highest with oxygen in both experiments. Similarly the percentage ratio of free to bound activity in the rudiments was highest in oxygen.

b) β -glucuronidase.

The results from two experiments, each using 48 paired rudiments, for comparison of the same gas phases are shown in Table 13. Unlike the acid phosphatase results, the media levels were nearly equal at two days, but became elevated in oxygen at four days. As with phosphatase the highest levels in the media were obtained in air at six days. The total activities in oxygen were different in the two experiments, exceeding that in air in one and being lower in the other. However, the percentage of free enzyme activity in the medium was still highest with

TABLE 12

ESTIMATION OF ACID PHOSPHATASE IN RUDIMENTS AND MEDIA.

	Experiment 1				Experiment 2			
	Air A.	Air B.	Oxygen	HBO	Air A.	Air B.	Oxygen	HBO
MEDIA 2 days	0.05	0.05	0.20	0.09	0.09	0.14	0.27	0.20
4 days	0.15	0.17	0.15	0.14	0.05	0.05	0.09	0.09
6 days	0.51	0.45	0.25	0.33	0.68	0.54	0.30	0.29
TOTAL	0.71	0.67	0.60	0.56	0.82	0.73	0.66	0.58
RUDIMENTS FREE	0.75	0.62	0.35	0.36	0.62	0.54	0.38	0.38
BOUND	0.62	0.90	0.21	0.39	0.56	0.56	0.23	0.59
TOTAL	1.37	1.53	0.56	0.75	1.18	1.10	0.61	0.97
% Activity in Medium	34%	30%	52%	42%	41%	39%	50%	42%
% Free/Bound in Rudiments	121%	69%	167%	92%	110%	97%	166%	64%

All enzyme levels expressed in Sigma units. (1 unit liberates 1 μM of p-nitrophenol per hour.)

Each measurement obtained from 12 rudiments paired as follows:- Air A - OXYGEN
Air B - HBO

TABLE 13

ESTIMATION OF β-GLUCURONIDASE IN RUDIMENTS AND MEDIA.

	Experiment 1				Experiment 2			
	Air A	Air B	Oxygen	HBO	Air A	Air B	Oxygen	HBO
MEDIA 2 days	67	46	67	100	76	61	70	55
4 days	18	21	168	100	12	10	32	18
6 days	104	112	30	31	102	108	38	32
TOTAL	189	189	265	231	220	179	140	105
RUDIMENTS FREE	145	122	52	61	115	108	33	48
BOUND	35	33	18	29	24	45	16	21
TOTAL	180	155	70	90	139	153	49	69
% Activity in Medium	51%	55%	79%	72%	61%	54%	74%	60%
% FREE/Bound in Rudiments	414%	370%	289%	210%	479%	240%	206%	229%

All enzyme levels expressed in standard units (20 units = 1 μgm Phenolphthalein liberated per hour).

Each measurement obtained from 12 rudiments paired as follows:- Air A - OXYGEN
Air B - HBO

oxygen in both experiments suggesting that more was released under these conditions. The total activity in the rudiments was again lowest in oxygen, but unlike the acid phosphatase the percentage ratio of free to bound activity within the rudiments was lower in oxygen than in air.

c) Acid protease.

Table 14 shows the results from one experiment comparing the enzyme activity in rudiments and media for the three gas phases. In oxygen there was an elevated enzyme level in the media at two days and the total activity over the six day period of culture was higher in this gas than in the controls in air. Similarly the total enzyme activity in the rudiments was lower with oxygen or hyperbaric oxygen, than with air. The highest percentage activity of enzyme in the media was obtained with oxygen and the percentage ratio of free to bound activity in rudiments was very much higher in oxygen when compared with the controls in air.

d) Effect of Hydrocortisone on Acid Protease.

The effect of adding hydrocortisone to the medium in a dosage of 1.0 μgm/ml on the production and release of lysosomal enzymes is shown in Table 15. In a single experiment with a gas phase of 95% air the enzyme activities in the media were roughly equal over the six day period of culture. The free and bound activity in the rudiments was also similar and seemed unaffected by the addition of hydrocortisone. The percentage of the total enzyme activity in the media was reduced by 10% with the addition of hydrocortisone, but the ratios of free to bound activity in the rudiments were virtually equal.

TABLE 14

ESTIMATION OF ACID PROTEASE (CATHEPSIN D) IN RUDIMENTS AND MEDIA.

	Air A	Air B	Oxygen	HBO
MEDIA 2 days	22.0	26.0	41.0	25.0
4 days	0	4.5	6.0	2.0
6 days	4.5	7.0	3.0	0
TOTAL	26.5	37.5	50.0	27.0
RUDIMENTS FREE	2.0	1.0	19.5	19.0
BOUND	29.5	30.0	7.0	7.5
TOTAL	31.5	31.0	26.5	26.5
% Activity in Media	46%	55%	65%	50%
% FREE/Bound in Rudiments	7%	3%	279%	253%

All enzyme levels expressed in standard units
(1 unit = 10^{-6} mEq of Tyrosine)

Each measurement obtained from 12 rudiments paired as follows:-

Air A - OXYGEN

Air B - HBO

TABLE 15

EFFECT OF HYDROCORTISONE 1.0 μgm/ml ON ACID PROTEASE IN RUDIMENTS AND MEDIA.

	Experiment 1		Experiment 2		Experiment 3		Experiment 4		Experiment 5	
Gas Phase	Air	Air +HC	O_2	O_2 +HC	O_2	O_2+ HC	HBO	HBO + HC	HBO	HBO + HC
MEDIA 2 days	32.0	26.0	52.5	24.0	23.5	40.0	11.0	4.5	6.0	2.5
4 days	3.0	7.5	2.5	7.0	67.0	30.0	10.0	5.0	17.5	14.0
6 days	10.0	10.0	4.0	4.0	5.0	37.0	0	11.5	10.0	0
TOTAL	45.0	43.5	59.0	35.0	95.5	107.0	21.0	21.0	33.5	16.5
RUDIMENTS FREE	27.0	28.0	17.0	40.0	33.0	83.0	12.0	17.5	11.0	13.5
BOUND	60.0	69.0	19.5	63.0	4.0	28.0	6.5	6.5	4.0	2.5
TOTAL	87.0	97.0	36.5	103.0	37.0	111.0	18.5	24.0	15.0	15.5
% Activity in media	43%	31%	62%	25%	72%	49%	53%	46%	69%	51%
% FREE BOUND in rudiments	45%	41%	87%	63%	825%	296%	185%	262%	275%	540%

Each measurement represents enzyme production or release by 12 rudiments.

The influence of hydrocortisone in the two experiments with a gas phase of 95% oxygen was more marked. The media levels of enzyme were similar, but when this was taken as a percentage of the total activity the hydrocortisone produced a fall of 20 - 30%. The total enzyme content of the rudiments was much higher in the hydrocortisone group with a corresponding reduction in the ratio of free to bound activity. These results are consistent with a stabilising effect of hydrocortisone on the lysosomal membranes.

In the experiments using 98% oxygen at hyperbaric pressure the media levels again did not differ widely. The percentage of total activity in the media was only reduced by approximately 10% as seen with the rudiments in air. The total enzyme levels in the rudiments were equal but the ratio of free to bound activity was surprisingly increased by the addition of hydrocortisone to the medium.

2) Growth in length.

a) Effect of Hydrocortisone.

In the 'late' tibial rudiments used for the majority of the histological studies, hydrocortisone added to the media in a dosage of 1.0 µg/ml or 5.0 µg/ml produced no significant alteration in the percentage elongation with any of the three gas phases.

In one experiment using 36 'early' rudiments, which consisted largely of cartilage at the time of explantation, growth on the medium containing 1.0 µg/ml of hydrocortisone exceeded that on the plain medium. This occurred in the early

stages of culture in air, but by six days the elongation in the two groups of rudiments was equal. In 95% oxygen and hyperbaric oxygen a similar early effect was seen, but at six days the increase was still greater with hydrocortisone by about 1%. The detailed results are shown in Table 16.

b) Effect of EACA.

The addition of EACA to the medium in a concentration of 0.1M was only used with 'late' tibial rudiments and produced no demonstrable alteration in elongation when compared with controls. The relative lengths of shaft bone to end cartilages were also measured for these rudiments, but no alteration occurred in this ratio with any of the gas phases used.

3) Histological structure.

a) Effect of hydrocortisone.

In the 64 pairs of 'late' tibial rudiments used to study the influence of hydrocortisone on the histological changes produced by alteration of the gaseous phase, no detectable difference was observed between the two concentrations used. In all experiments the observed pH of the medium containing hydrocortisone was on average 0.05 more alkaline than the control at each media change.

Rudiments cultured in air showed little difference after one day, but at two days those exposed to hydrocortisone demonstrated alterations in both bone and cartilage. The end cartilages (Fig. 61) showed denser metachromatic staining in the proliferative and maturation layers, though there was some central lysis present. The perichondrium was slightly thickened at both ends and showed some positive PAS staining compared

TABLE 16

PERCENTAGE INCREASE IN LENGTH OF 'EARLY' RUDIMENTS WITH HYDROCORTISONE 10 μg/ml.

		PLAIN MEDIUM		MEDIUM + HYDROCORTISONE	
		% increases	Mean ± S.D.	% increases	Mean ± S.D.
AIR	2 day	12.3, 6.5, 9.1	9.3 ± 2.9	15.4, 19.6, 15.4	16.8 ± 2.4
	4 day	13.8, 9.7, 11.5	11.7 ± 2.0	16.9, 19.4, 13.1	16.5 ± 3.2
	6 day	26.1, 9.7, 13.0	16.3 ± 8.7	21.5, 18.0, 7.8	15.8 ± 7.1
OXYGEN	2 day	3.0, 6.7, 14.0	7.9 ± 5.6	18.3, 6.5, 9.8	11.5 ± 6.0
	4 day	14.7, 16.7, 3.8	11.7 ± 6.9	18.3, 12.9, 15.4	15.5 ± 2.3
	6 day	14.7, 16.7, 3.7	11.7 ± 7.0	13.3, 14.5, 11.1	12.9 ± 1.7
HBO	2 day	3.3, 6.6, 3.8	4.6 ± 1.7	13.1, 14.5, 12.0	13.2 ± 1.2
	4 day	1.7, 8.2, 5.7	5.2 ± 3.3	8.2, 6.5, 3.8	6.2 ± 2.2
	6 day	8.3, 8.2, 3.8	6.7 ± 2.5	8.2, 9.7, 3.7	7.2 ± 3.1

with controls. Despite this there was no lateral invasion into the junctional zone between the proliferative and hypertrophic cells with flattening of the chondrocytes and positive azan and PAS staining. After six days this was well established in controls but still absent in the rudiments on hydrocortisone. The bone of the shaft (Fig. 62) showed smaller, denser osteoblasts and osteocytes with little evidence of endosteal resorption. The reticular cells of the marrow cavity were also smaller, denser and more homogenous than the varied cell population in controls.

In 95% oxygen at normobaric pressure the hydrocortisone inhibited the appearance and resorptive activity of osteoclasts in the metaphyseal region after one day in culture (Figs. 63A and B). After two days the effect of this was even more marked (Figs. 64A and B) with almost total resorption of the distal hypertrophic cells in plain media, but with preservation of most of these chondrocytes on hydrocortisone. Differences were also apparent in the shaft bone (Figs. 65A and B) with less evidence of osteocytic lacunar enlargement and marked thickening in the periosteal layer of hydrocortisone treated rudiments. The osteoblastic cells in the deeper layers appeared more spindle shaped and fibroblastic than the normal osteogenic cells seen in air. However, after four and six days the rudiments showed fewer differences with widespread osteolysis of the shaft bone, thinning of the periosteum, and loss of marrow cells occurring in both groups. The hydrocortisone rudiments still showed better preservation of hypertrophic chondrocytes and endochondral trabecular bone, though some resorption occurred.

The effects of hyperbaric oxygen were less modified by hydrocortisone. After one day both sets of rudiments showed the presence of a few osteoclasts in the metaphyseal region (Figs. 66A and B). These failed to produce any significant resorption and at two days both groups showed well maintained hypertrophic zones with vertical septa continuous with some of the cartilaginous cores of the endochondral bone trabeculae. The slight thickening in the perichondrium and periosteum seen in the first 48 hours of exposure to hyperbaric oxygen was abolished by hydrocortisone, though the cells were still small with pyknotic nuclei. The toxic changes seen in the cells during the late stages of exposure to hyperbaric oxygen at four and six days were not inhibited to any extent by hydrocortisone. However, what was apparent in the end cartilages was a lessened hydration of the chondrocytes in all layers with a retention of strong ground substance metachromasia.

A few ' early' cartilaginous rudiments were examined histologically in view of the increased elongation reported with hydrocortisone. In all three gas phases a marrow cavity developed (Figs. 67A to D) after only two days in culture, but there was some variation in the extent of hypertrophic chondrocyte resorption. In 95% oxygen the cavity was poorly developed and there was a marked increase in the quantity of new bone formed in the deeper layers of the periosteum, which occupied the space between the end cartilages in the hydrocortisone rudiments (Fig. 68C). The superficial layers of the periosteum were also more thickened than with air or hyperbaric oxygen, though the cell nuclei were more rounded. In hyperbaric oxygen the

amount of periosteal bone formed was no greater than with air (Figs. 68B and 68D), while the superficial periosteal cells showed smaller, denser nuclei.

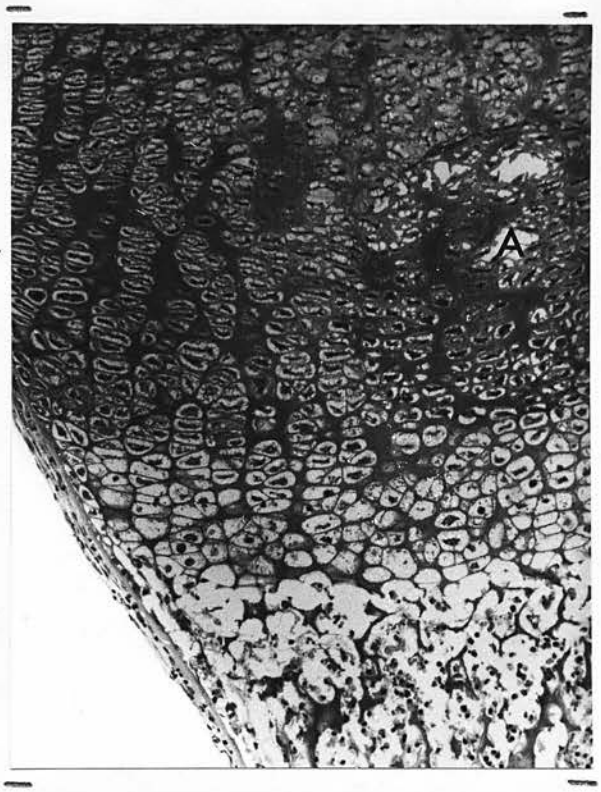
After four to six days in culture the most marked feature in all hydrocortisone rudiments, particularly with those exposed to both types of hyperoxia, was an elongation of the end cartilages from cell proliferation in the maturation and hypertrophic zones with little evidence of resorption at the centre of the shaft. In 95% oxygen a large quantity of bone continued to fill the gap between the end cartilages, but there was less thickening of the perichondrium than with plain media. The metachromasia on the end cartilage was preserved by hydrocortisone in both conditions of hyperoxia, without the increase in PAS-positive staining seen on plain medium. In air the lateral invasion from the perichondrium failed to occur, though even in the control rudiments this was not as marked a feature as in the 'late' rudiments previously studied.

b) Effect of EACA.

The effect of 0.1M EACA on histology was only investigated with 'late' tibial rudiments. In air it slowed, but did not prevent, the loss of metachromasia in the end cartilages associated with the lateral erosion from the perichondrium. However, the superficial cells in the perichondrium and periosteum appeared smaller and less healthy suggesting that the EACA might have some toxic effect. It had little effect on the changes in the bony shaft during the period of culture and the formation of a layer of osteoblasts partially sealing the end cartilages occurred in both sets of rudiments.

In 95% oxygen (Figs. 69A and B) EACA did not inhibit the appearance of osteoclasts in the metaphyseal region, nor their resorption of the hypertrophic cell layer. It also failed to reduce the osteocytic osteolysis and reduction in marrow cell population seen after four to six days in culture, though it did preserve slightly better metachromasia in the end cartilages.

The effect of EACA on rudiments exposed to hyperbaric oxygen was not marked. After one day it did permit more thickening in the periosteal layer, though the superficial cells still showed small, dense nuclei (Figs. 70A and B). There was some reduction in the amount of trabecular bone resorption, but in the later stages of culture the generalised osteolysis and toxic effects of hyperbaric oxygen were equally apparent in both groups of rudiments.

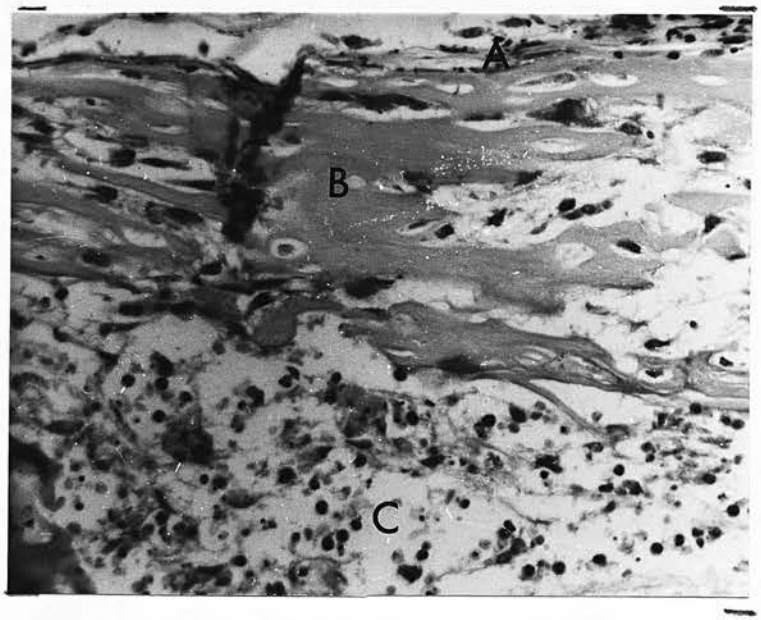


A. Chondrocyte lysis with loss of metachromasia

x 90

Alcian Blue & PAS

Fig. 61. End cartilage after 2 days in Air and Hydrocortisone.



A. Periosteum with small osteoblasts

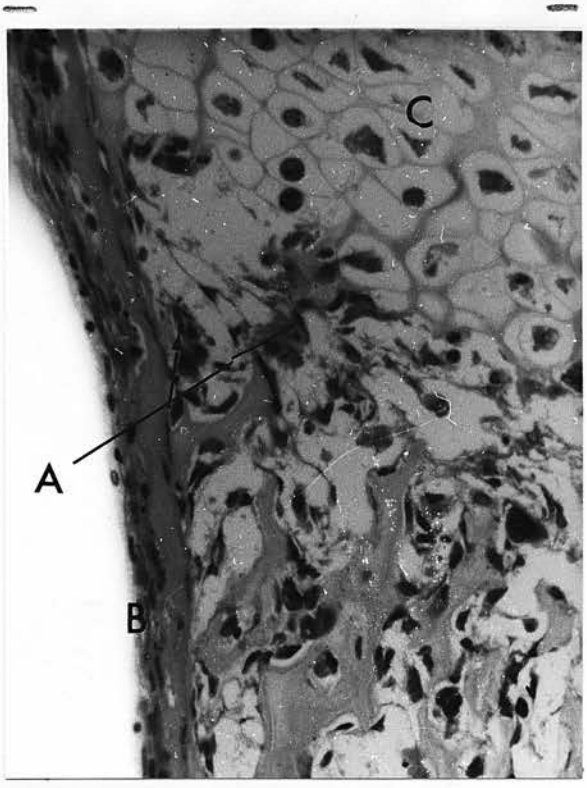
B. Bone

C. Marrow cavity

x 220

Alcian Blue & PAS

Fig. 62. Shaft bone after 2 days in Air and Hydrocortisone.



A. Osteoclasts

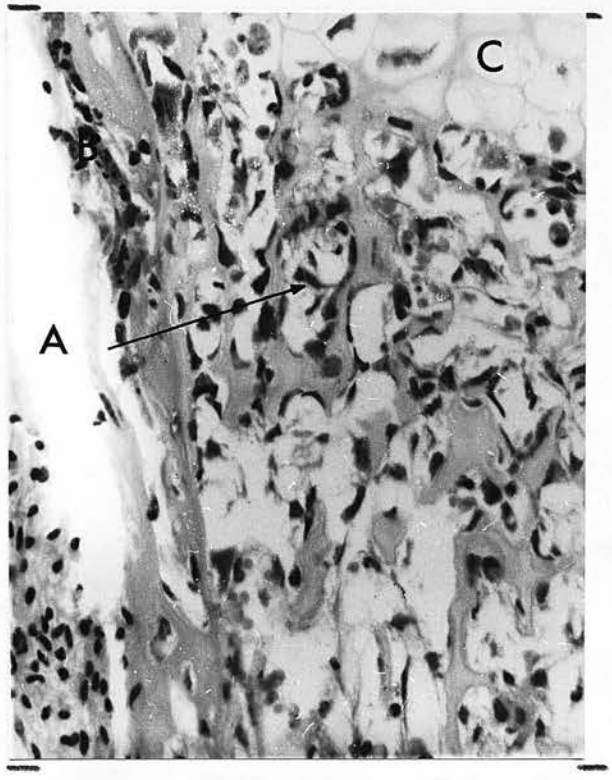
B. Periosteum

C. Hypertrophic chondrocytes

H. & E.

x 220

Fig. 63A. Metaphysis after 1 day in Oxygen.



A. Macrophages

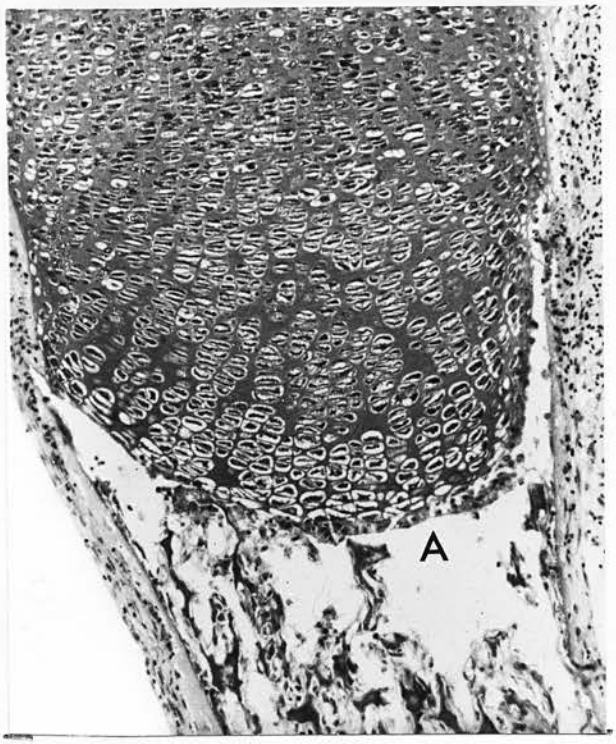
B. Periosteum

C. Hypertrophic chondrocytes

H. & E.

x 220

Fig. 63B. Metaphysis after 1 day in Oxygen + Hydrocortisone.

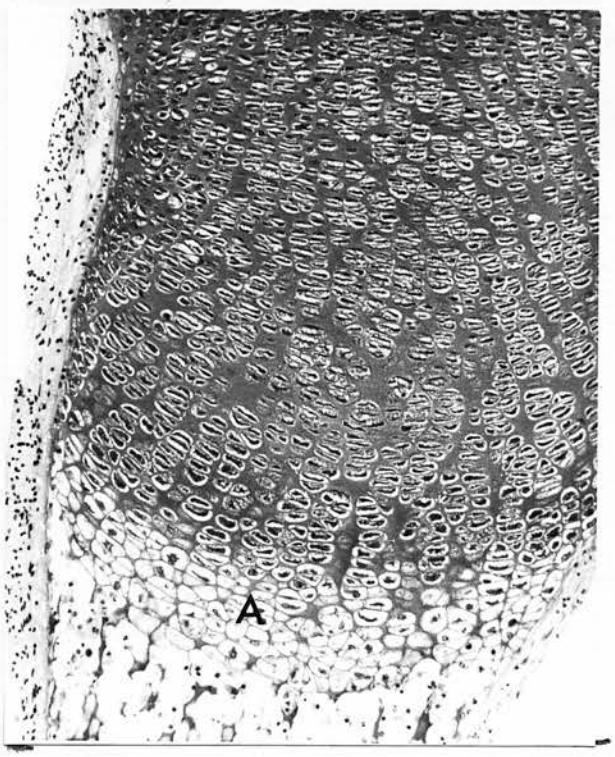


A. Site of resorption of hypertrophic chondrocytes

x 90

Alcian Blue & PAS

Fig. 64A. End cartilage after 2 days in Oxygen.

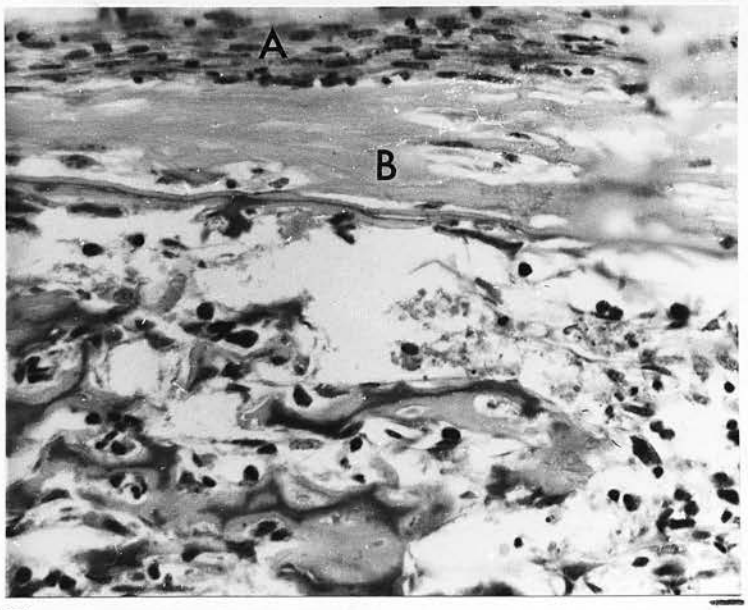


A. Hypertrophic chondrocytes

x 90

Alcian Blue & PAS

Fig. 64B. End cartilage after 2 days in Oxygen + Hydrocortisone.



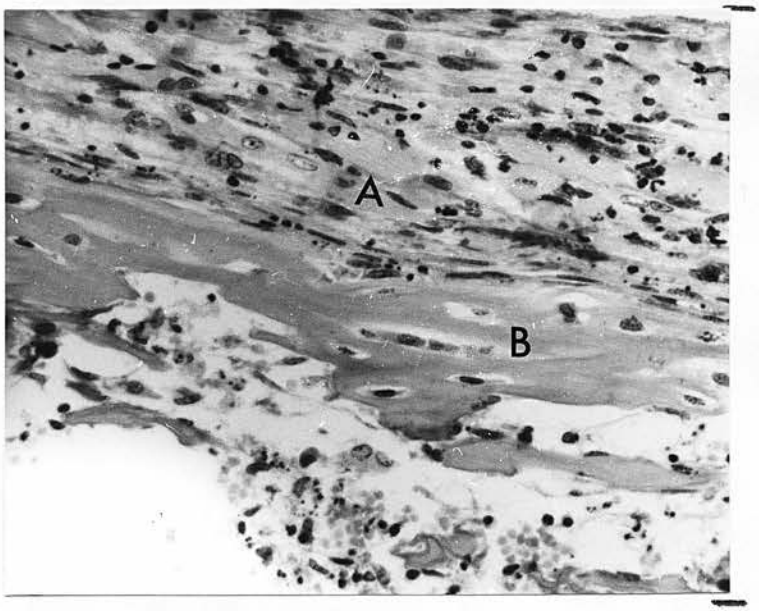
A. Periosteum

B. Bone with early osteocytosis

x 220

Alcian Blue & PAS

Fig. 65A. Shaft bone after 2 days in Oxygen.



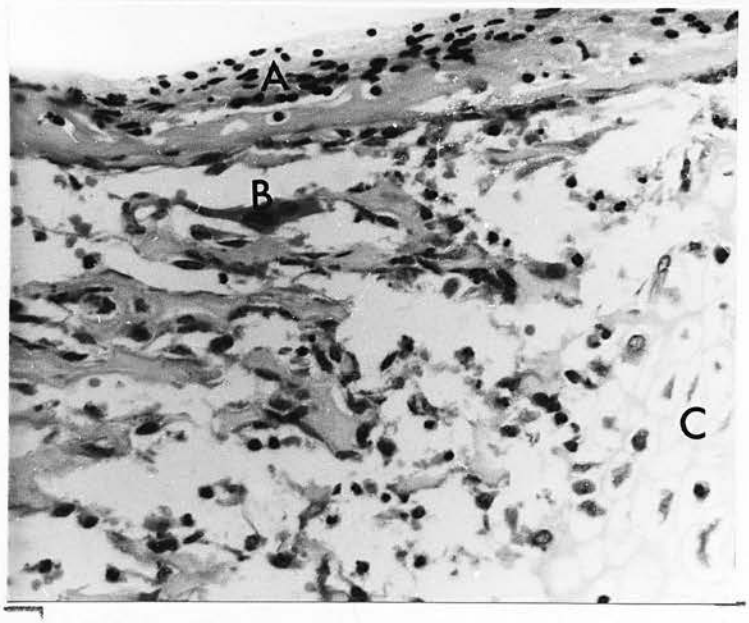
A. Periosteum (thickened)

B. Bone with intact osteocytes

x 220

Alcian Blue & PAS

Fig. 65B. Shaft bone after 2 days in Oxygen + Hydrocortisone.



A. Periosteum (small cells)

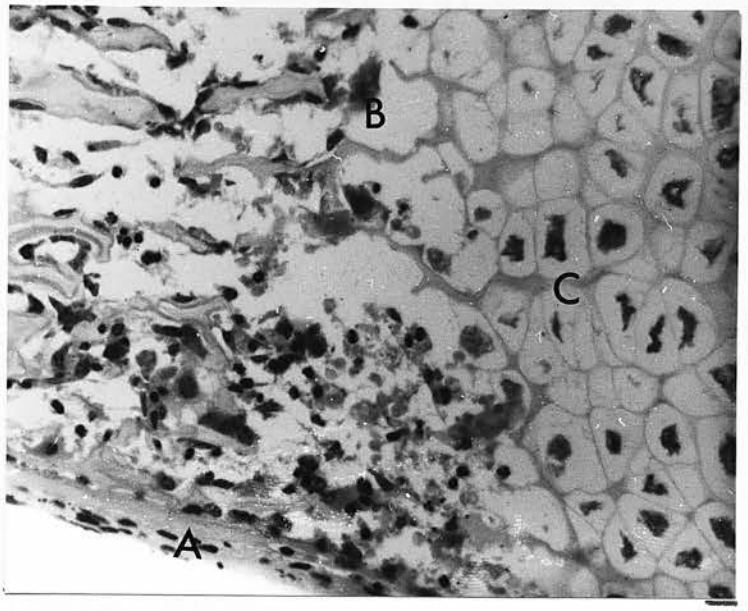
B. Osteoclasts

C. Hypertrophic chondrocytes

x 220

Alcian Blue & PAS

Fig. 66A. Metaphysis after 1 day in Hyperbaric Oxygen.



A. Periosteum (small cells)

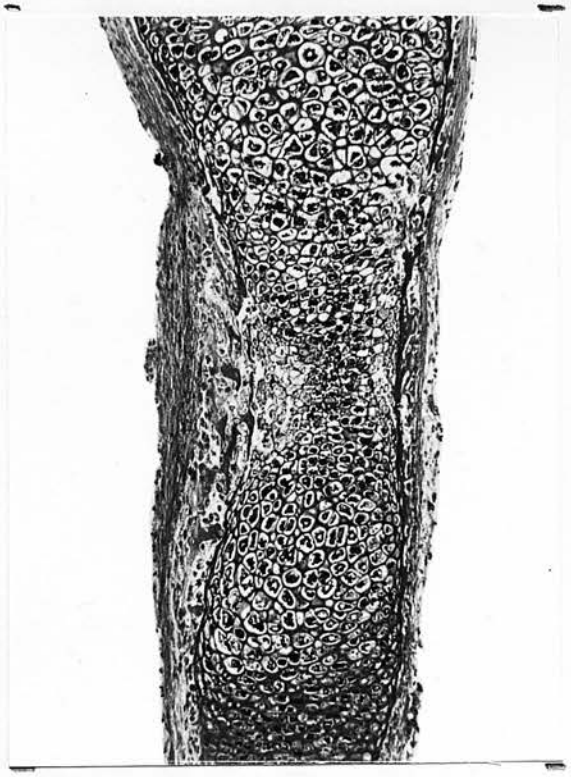
B. Osteoclasts

C. Hypertrophic chondrocytes

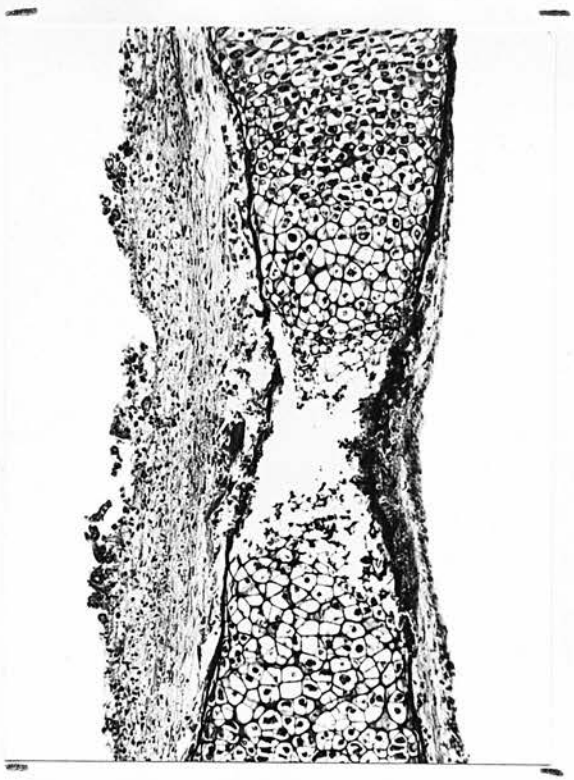
x 220

Alcian Blue & PAS

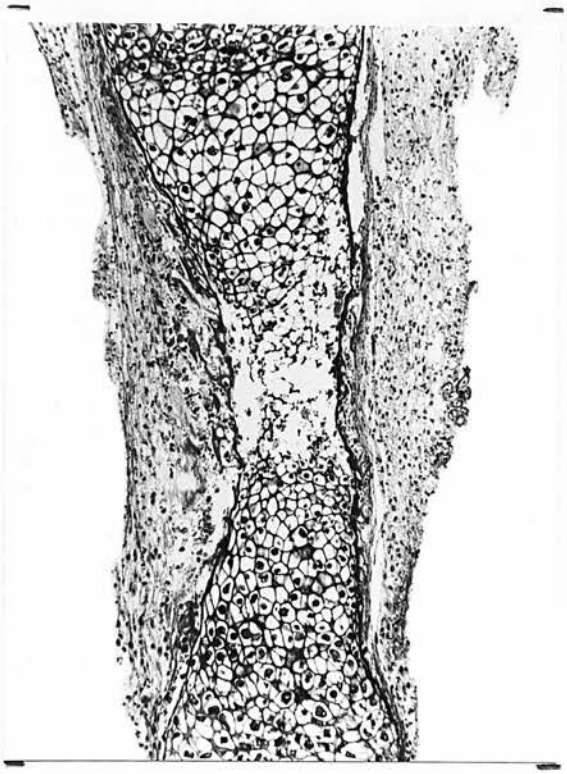
Fig. 66B. Metaphysis after 1 day in Hyperbaric Oxygen + Hydrocortisone.



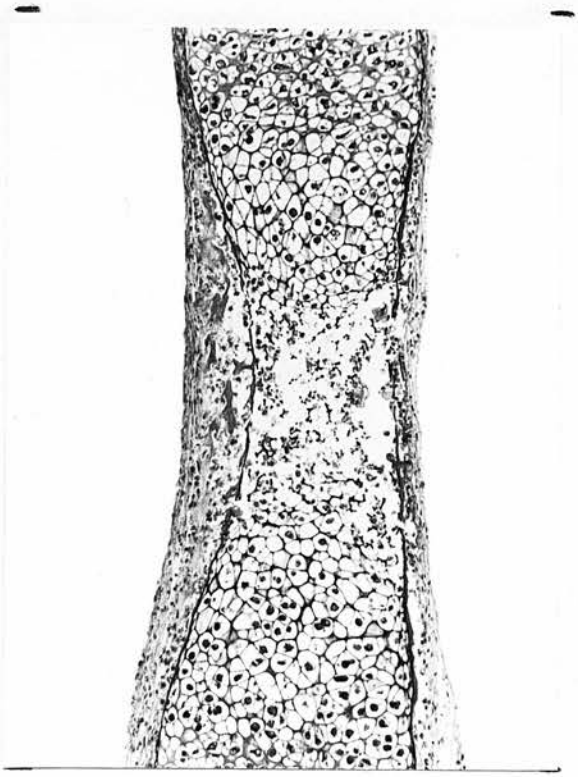
A. Zero control



B. Air + Hydrocortisone



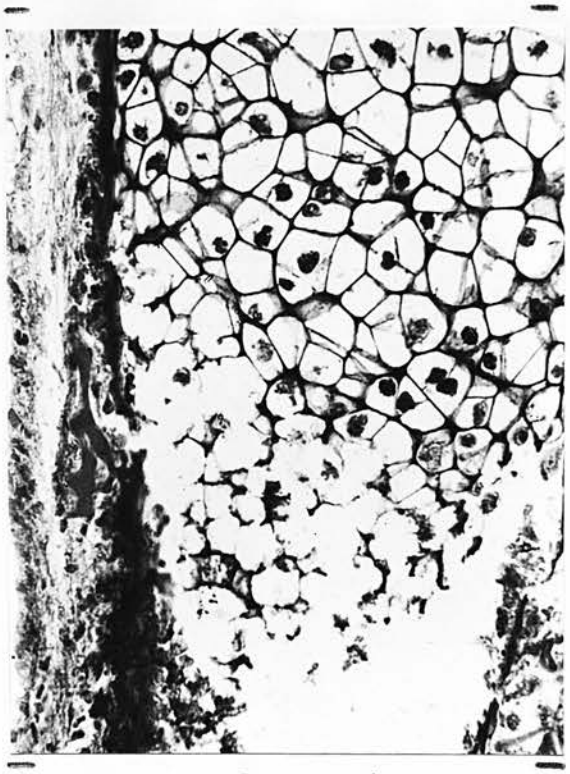
C. Oxygen + Hydrocortisone



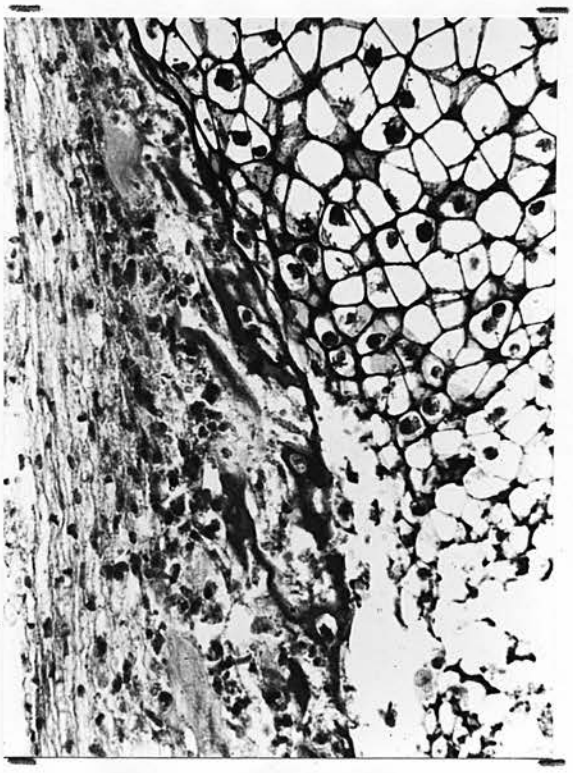
D. HBO + Hydrocortisone

Fig. 67A-D. Early rudiments to show formation of marrow cavity after 2 days in culture. x 125. Alcian Blue & PAS.

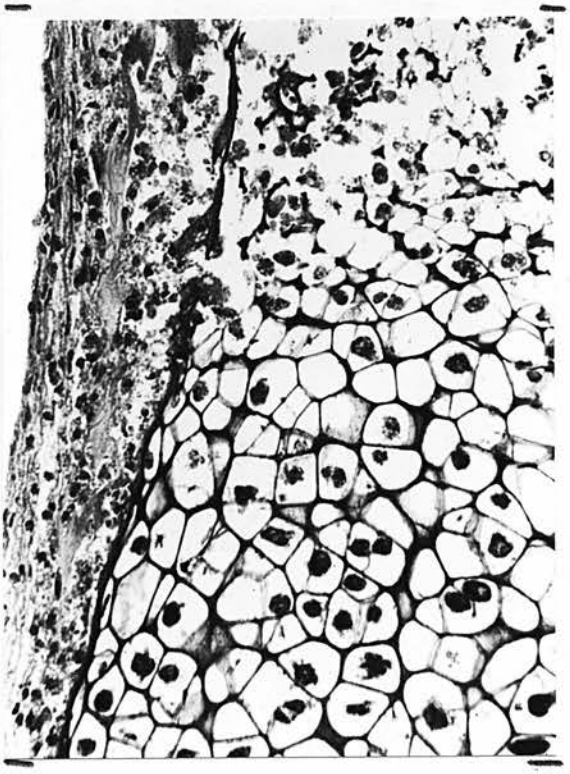
A. Zero control



B. Air + Hydrocortisone

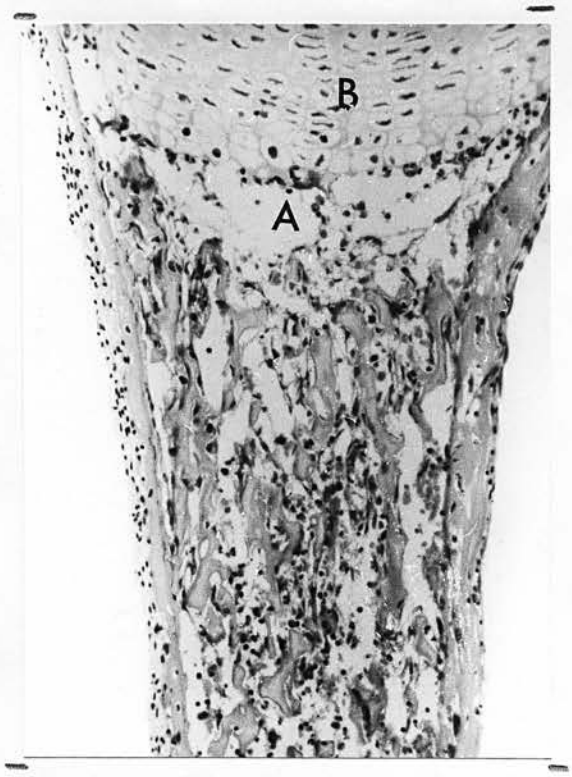


C. Oxygen + Hydrocortisone



D. HBO + Hydrocortisone

Fig. 68A-D. Periosteal Bone formation in early rudiments after 2 days in culture. x 350. Alcian blue & PAS.



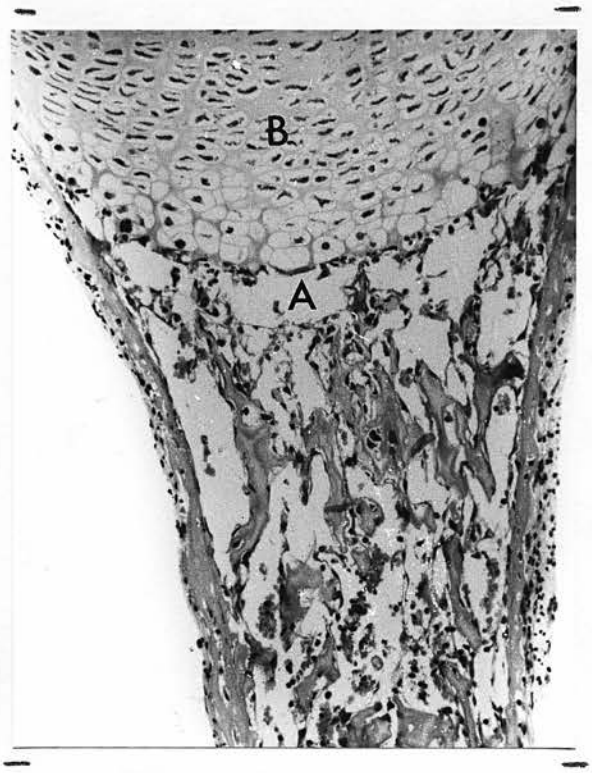
A. Site of osteoclastic resorption

B. End cartilage

H. & E.

x 90

Fig. 69A. Metaphysis after 1 day in Oxygen.



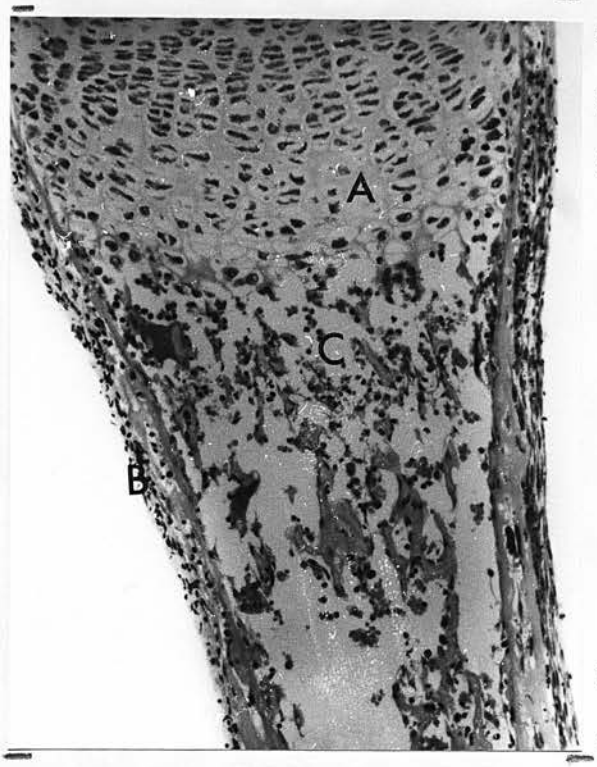
A. Site of osteoclastic resorption

B. End cartilage

H. & E.

x 90

Fig. 69B. Metaphysis after 1 day in Oxygen + EACA.



A. End cartilage

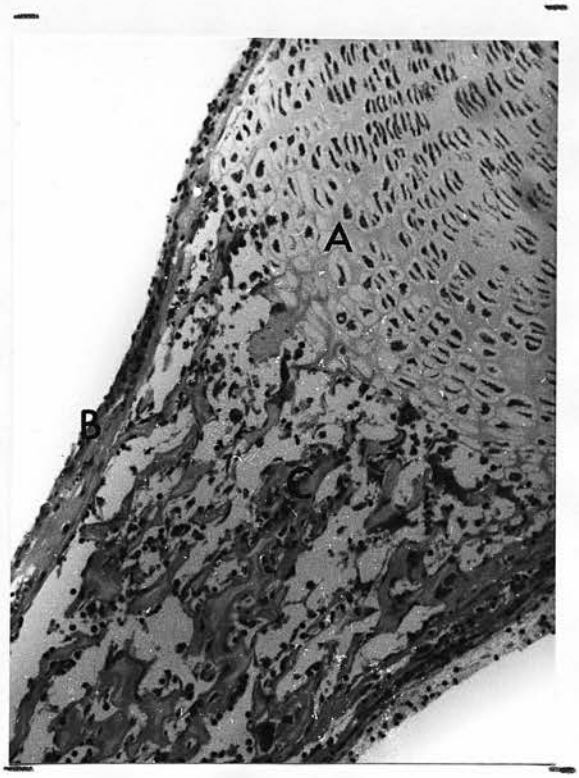
B. Periosteum

C. Endochondral bone resorption

H. & E.

x 90

Fig. 70A. Metaphysis after 1 day in Hyperbaric Oxygen.



A. End cartilage

B. Periosteum

C. Endochondral bone

H. & E.

x 90

Fig. 70B. Metaphysis after 1 day in Hyperbaric Oxygen + EACA.

DISCUSSION

1) Production and release of lysosomal enzymes.

The effects of hyperoxia on the production and release of the three lysosomal enzymes studied were somewhat variable. This could have resulted from variables in the experimental technique, particularly in regard to the homogenisation of rudiments. Any extensive resorption in the rudiments producing overall softening would make the homogenisation techniques more effective and could in itself result in increased intracellular enzyme release. The need to use twelve rudiments on each grid to obtain measurable levels of enzyme activity in the media might also have introduced a problem of inadequate nutrition for the tissues because of the small volume of 1.5 ml used in each dish.

The release of acid phosphatase into the medium was enhanced by normobaric hyperoxia after two days, but the total media activity was still highest in air over the total culture period. One possible explanation would be a failure of serum to bind the enzyme activity released in the first twenty-four hours, when from the histological studies of Part III osteoclastic resorptive activity appeared to be at a maximum. This does not explain the high levels in air at six days. This may reflect the onset of degenerative autolysis in the rudiments, although morphologically this was never as great as in oxygen at two days, or could be related to the changes in the end cartilages associated with the appearance of secondary ossification centres. Despite this the percentage of total acid phosphatase activity in the medium was still highest in hyperoxia at atmospheric pressure.

This also proved true with β-glucuronidase and acid protease. Exposure to hyperbaric oxygen did not increase the percentage of phosphatase activity in the medium to the same extent. The low levels obtained in the media and rudiments under this condition would be consistent with the morphological changes previously observed. The early pyknosis and nuclear death may not allow the liberation of enzymes, even in the presence of degeneration.

β-glucuronidase behaved differently to the other two enzymes showing an elevated media level in hyperoxia at four days and a higher total release over the period of culture compared to the controls in air. This fits the findings of Tolnai (1968) who reported increased release, but not synthesis, of this enzyme in bone resorption. Certainly the rudiment enzyme content was lowest with 95% oxygen, while in addition the ratio of free to bound activity in the rudiments was not elevated as much as with the other two enzymes.

The acid protease results ran more parallel with the acid phosphatase, showing the highest percentage of release into the medium with 95% oxygen. The ratio of free to bound activity in the rudiments was also elevated with both types of hyperoxia, suggesting that these enzymes have a similar origin and mechanism of release.

2) Effect of Hydrocortisone on Acid Protease production and release.

Hydrocortisone in a dosage of 1.0 μgm/ml had little effect on the release of acid protease into the medium with 95% air. It did not reduce the slightly elevated levels which occurred

between four and six days in culture suggesting that these were not due to a loss of lysosomal membrane stability. It also failed to influence the ratio of free to bound activity in the rudiments which would confirm this fact.

With 95% oxygen at atmospheric pressure the hydrocortisone had a definite effect in reducing the free media activity and the ratio of free to bound enzyme activity in the rudiments. This would support the findings of Sledge and Dingle (1965) who reported similar results with embryonic chick cartilage exposed to hyperoxia. The stabilising effect on lysosomal membranes was not evident in the experiments with hyperbaric oxygen although there was some reduction of the percentage release of enzyme into the medium. The increased ratio of free to bound enzyme activity in the rudiments with hydrocortisone might indicate that it was able to hold the enzyme in the tissues before the more generalised toxic effects of the increased oxygen pressure became evident. These results will be reassessed in relation to the histological findings with lysosomal enzyme inhibitors and blocking agents.

3) Effect of Hydrocortisone on growth and structure of rudiments.

The failure of Hydrocortisone to influence elongation of the 'late' rudiments is not surprising in view of their large ratio of bone shaft to end cartilages. This finding is in agreement with most other workers and would fit the suggestion that cartilage is more susceptible to the action of lysosomal enzymes than is bone. The results with the 'early'rudiments consisting largely of cartilage, were somewhat surprising as previous work has suggested that hydrocortisone inhibits cell division and therefore elongation of the rudiments. Histologically

there was undoubted proliferation of the chondrocytes and the effect of hydrocortisone in inhibiting their resorption probably accounts for this discrepancy. The failure to develop such a large marrow cavity as in control rudiments prevented some of the central collapse which occurred in these and might explain their relatively shorter length.

The hydrocortisone certainly inhibited resorption of cartilage both at the distal row of the hypertrophic cells and by the perichondrium into the lateral erosion bays described by Fell and Thomas (1961). This would suggest that both mechanisms are dependent on the release of lysosomal enzymes, although other possible mechanisms could be suggested. The inhibition of osteoclastic activity in normobaric hyperoxia could result from the arrested differentiation of this type of cell. Certainly other inhibitory effects of hydrocortisone on cell morphology were seen with small immature fibroblastic periosteal cells and a depression of the marrow cell series. It was also noted that all the media containing hydrocortisone was slightly more alkaline and though this still lay within physiological limits it might depress the effect of lysosomal enzymes which are active at an acid pH. The other effects of the hydrocortisone would fit a mechanism of lysosomal membrane stabilisation since the end cartilage metachromasia was preserved together with more intact transverse septa in the hypertrophic cartilage zone.

The later effects of hyperoxia in the bony shaft with the production of osteocytic osteolysis were not inhibited by hydrocortisone suggesting that these are not dependent on lysosomal

enzyme release. The dose used could have been inadequate but the findings would also fit the suggestion of other workers such as Shimizu et al (1969) that a non-lysosomal enzyme such as collagenase is responsible.

Hydrocortisone also failed to control the toxic degenerative effects of hyperbaric oxygen, which in vivo work suggests are due to damage of the mitochondrial enzymes. Despite this hydrocortisone did permit a slight increase in the periosteal thickening and new bone formation in the early stages of exposure to elevated oxygen concentration of pressure. Whether this could be maintained by intermittent exposure to hyperoxia combined with hydrocortisone requires further investigation. It would fit the suggestion of Reynolds (1966) that hydrocortisone allows an increase in collagen content of rudiments at the same time as their polysaccharide content falls.

4) Effect of EACA on growth and structure of rudiments.

This lysosomal enzyme inhibitor had no effect on elongation in the 'late' tibial rudiments used in these experiments. It would be interesting to know whether it could inhibit the relative shortening produced in the 'early' rudiments exposed to hyperoxia. The inhibition of osteoclastic activity reported by Sledge and Dingle (1965) in the chick bone and by Brighton and Schaffzin (1970) in mouse bone was not seen here. This could be explained by a species difference or alternatively could be the result of a lower dosage. Further experiments would be required using an increased 0.5M concentration. The suggestion of inadequate dosage would also be supported by the finding of preserved lateral invasion of the perichondrium into the end

cartilages in rudiments in air. This was partly inhibited, but could represent a process which is not entirely dependent on the release of lysosomal enzymes or their activity.

GENERAL DISCUSSION AND SUGGESTED FURTHER WORK

GENERAL DISCUSSION AND SUGGESTED FURTHER WORK.

The choice of the embryonic mouse limb bone rudiment for the study of the effects of varying oxygen concentration and pressure on skeletal tissues was only partially satisfactory. In the later stages of development it provides both bone and cartilage tissues, with periosteal and endochondral ossification. Under the most optimum culture conditions both these processes continue, though at a very reduced rate compared with the in vivo situation. The main variables affecting the growth and survival of rudiments in culture were the medium and the gas phase. Considerable variation was seen between the synthetic media tested, with a high ascorbate level appearing to be a necessity, although none proved satisfactory without serum supplements. Even with heat inactivation this introduces unknown biological activity, which could be further enhanced by hyperoxia. This aspect requires further study and in future work the possibility of substituting albumen for serum will be investigated.

The morphological and biochemical changes induced in the rudiments by hyperoxia were only investigated over a limited range. The high normobaric concentration of 95% oxygen produced predominantly resorption and degenerative changes associated with osteoclastic activation and lysosomal enzyme release. Any stimulus of oxygen to osteogenesis was masked by these effects and there is a need for further experiments using intermediate concentrations between air and this high level. However, the organ culture technique can never allow

the variation in oxygen tensions at different sites in the bone and cartilage which occurs with the normal circulation from the nutrient artery. This seems to be the requirement for the normal development of the growth plate and ordered endochondral ossification. The embryonic rudiments exhibit some features of self differentiation with the development of a marrow cavity and the early stages of secondary ossification centres, but this can be modified by the culture conditions and is never as complete as in vivo.

Hyperbaric oxygen at a pressure of two atmospheres absolute quickly produced toxic effects and eventual death in the rudiments. The experimental results suggest that in the early stages there may be some stimulus to periosteal bone formation, though this could be the result of either the increased pressure or dissolved oxygen concentration. Further experiments are suggested to expose the rudiments to a gas mixture which will give the same oxygen concentration as in air but at an increased pressure of two atmospheres. A study is also required to determine the effects of short intermittent exposures to hyperbaric oxygen, which might allow reversibility of the toxic effects and induce the cumulative stimulatory effects to osteogenesis suggested by previous clinical studies.

The mechanism of bone resorption in hyperoxia seems a complex one and cannot be fully explained by increased osteoclasia with associated release of lysosomal enzymes. It is not certain if the osteocytic osteolysis, which is prominent in the later stages, is accompanied by the same release of enzymes.

Nor is this necessarily in any way related to the physiological mechanism in vivo, which may require different enzyme systems. The new collagen formed during culture was totally resorbed, but as this is not calcified under these conditions its resistance would be less. Short term studies will be required at twelve hour intervals or less to correlate morphological changes, the incorporation and release of labelled proline into collagen, and the production and release of lysosomal enzymes. Another possible technique would be to pre-label the pre-explantation collagen in utero by administering another labelled amino-acid to the mother. Because of the difficulty in producing adequate media levels for measurement this may need an alternative model, such as the mouse calvarium, which provides a much greater mass of tissue but loses the cartilage to bone interaction.

The results reported here and any gained from future work with the same techniques have the drawback of all in vitro studies, which of necessity simplify the physiological mechanisms in vivo. They are only valid when interpreted against similar results obtained from experiments with the intact animal.

ACKNOWLEDGEMENTS.

This work was carried out with financial aid from a Medical Research Council grant, which is acknowledged with thanks. I am extremely grateful to my supervisor Professor J.I.P. James, M.S., F.R.C.S. for his continued interest and encouragement throughout this work.

The experimental work was carried out in the Department of Orthopaedic Surgery, University of Edinburgh and I am indebted to the technical staff of the department for their help. In particular, I was assisted in the preparation of histological material by Mr. R. Black and Mrs. Y. Hair, and in the biochemical estimations by Dr. M. Longstaff.

At the inception of this work I was fortunate to be instructed in the basic techniques of organ culture by Dame Honor Fell, F.R.S., and Dr. J. Reynolds at the Strangeways Laboratory, Cambridge. Their continued interest and advice was much appreciated.

I was assisted in the preparation of photographic material for the thesis by the staff of the medical photographic departments in Edinburgh and Glasgow. My particular thanks go to Mr. M. Devlin, Mr. J. Paul and Mr. J. Anderson.

Finally I extend my warm thanks to a series of patient and tireless secretaries in Edinburgh and Glasgow who have typed and retyped this work. They were Mrs. J. Heppel, Mrs. S. Lyle, Mrs. N. Thompson, Miss C. Davidson and Miss R. McCall.

REFERENCES

ALI, S.Y. (1964) Biochem. J. 93 : 611 - 618. The degradation of cartilage by an intracellular protease.

ALLISON, A.C., (1965) Nature (London) 205: 144 - 148. Role of Lysosomes in Oxygen Toxicity.

ANSON, M.L. (1938) J. Gen. Physiol. 22 : 79 - 89. The Estimation of Pepsin, Trypsin, Papain, and Cathepsin with Haemoglobin.

ASHER, M.A. & SLEDGE, C.B. (1968) Clin. Orth. 61 : 48 - 51. Hyperoxia and in-vitro bone resorption.

BARKA, T., & ANDERSON, P.J. (1962) J. Histochem. Cytochem. 10 : 741 - 753. Histochemical Methods for Acid Phosphatase using Hexazonium Pararosanilin as Coupler.

BARRETT, A.J. (1967) Biochem. J. 104 : 601 - 608. Lysosomal Acid Proteinase of Rabbit Liver.

BARRETT, A.J. (1969) "Properties of Lysosomal Enzymes" in Lysosomes in Biology and Pathology, Vol. 2., (ed. J.T. Dingle & Honor B. Fell), North Holland Publishing Co., Amsterdam, pp. 245 - 312.

BASSETT, C.A.L. (1962) J. Bone Jt. Surg. 44A : 1217 - 1244. Current Concepts of Bone Formulation.

BELANGER, L.F. (1965) "Osteolysis : An Outlook on its Mechanism and Causation", in The Parathyroid Glands, (ed. P.J. Gailliard & R.V. Talmage & A.M. Budy), University of Chicago Press, pp. 137 - 151.

BELANGER, L.F. (1969) Calc. Tiss. Res. 4 : 1 - 12. Osteocytic Osteolysis.

BELANGER L.F., & MIGICOVSKY, B.B. (1963) J. Histochem. Cytochem. 11 : 734 - 737. Histochemical Evidence of Proteolysis in Bone, The Influence of Parathormone.

BELANGER, L.F., ROBICHON, J., MIGICOVSKY, B.B., COPP, D.H., & VINCENT, J. (1963) "Resorption Without Osteoclasts (Osteolysis)", in Mechanisms of Hard Tissue Destruction, (ed. R.F. Sognnaes), Amer. Assoc. Advan. Sci., Washington, D.C. pp. 531 - 556.

BERGMAN, I. & LOXLEY, R. (1963) Anal. Chem. 35 : 1961 - 1965. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline.

BERNICK, S. (1971) Calc. Tiss. Res. 6 : 316 - 328. Histochemical Study of Bone in Parathyroidectomised Rats.

BEVELANDER, G., & JOHNSON, P.L. (1950) Anat. Rec. 108 : 1 - 22. A Histochemical Study of the Development of Membrane Bone.

BIGGERS, J.D. (1960) J. Exp. Zool. 145, : 227 - 242. The Growth in Dry Weight and Wet Weight of Embryonic Chick Tibiotarsi grown in Vivo and in Vitro.

BIGGERS, J.D., GWATKIN, R.B.L., & HEYNER, S. (1961). Exp. Cell. Res. 25 : 41 - 58. Growth of Embryonic Avian and Mammalian Tibiae on a relatively simple chemically defined medium.

BIGGERS, J.D., & HEYNER, S. (1963) J. Exp. Zool. 152 : 41 - 55. Growth of Embryonic Chick and Rat Long Bones in Vitro on Natural and Chemically Defined Media.

BIRGE, S.J., & PECK, W.A. (1966) Biochem. Biophis. Res. Commun. 22 : 532 - 539. Collagen Synthesis by Isolated Bone Cells.

BRIGHTON, C.T., & HEPPENSTALL, R.B. (1971) J. Bone Jt. Surg. 53A : 719 - 728. Oxygen Tension in zones of the epiphyseal plate, the metaphysis and diaphysis.

BRIGHTON, C.T., & SCHAFFZIN (1970) Calc. Tiss. Res. 6 : 151 - 161. Comparison of the effects of excess vitamin A and high oxygen tension on in vitro epiphyseal plate growth.

BRIGHTON, C.T., RAY, R.D., SOBLE, L.W., KUETTNER, K.E. (1969) J. Bone Jt. Surg. 51 : 1383 - 1396. In-vitro Epiphyseal Plate Growth in Various Oxygen Tensions.

BROOKES, M., & HELAL, B. (1968) J. Bone Jt. Surg. 50B : 493 - 504. Primary Osteoarthritis, Venous Engorgement and Osteogenesis.

BUNO, W., & GOYENA, H. (1955) Proc. Soc. Exp. Biol. & Med. 89 : 622 - 624. Effect of Cortisone upon growth in vitro of femur of the chick embryo.

BURSTONE, M.S., (1960) "Hydrolytic Enzymes in Dentinogenesis and Osteogenesis", in Calcification in Biological Systems, (R.F. Sognnaes), Am. Assoc. Advan. Sci., Washington, D.C., pp. 217 - 243.

CAMPO, R.D., & DZIEWIATKOWSKI, D.D. (1963) J. Cell. Biol. 18 : 19 - 29. Turnover of the Organic Matrix of Cartilage and Bone as visualized by Autoradiography.

CARNEIRO, J., & LEBLOND, C.P. (1959) Exp. Cell. Res. 18 : 291 - 300. Role of Osteoblasts and Odontoblasts in secreting the Collagen of bone and dentin as shown by Radioautography in Mice given tritium - labelled glycine.

CARTER, T.C. (1954) J. Genet. 52: 1 - 35. The Genetics of Luxate Mice IV. Embryology.

CHEN, J.M. (1954) Exp. Cell. Res. 7 : 518 - 529. The Cultivation in Fluid Medium of Organised Liver, Pancreas and other Tissues of Foetal Rats.

CHVAPIL, M., & HURYCH, J. (1968) "Control of Collagen Biosynthesis", in International Review of Connective Tissue Research, Vol. 4, (ed. David A. Hall), Academic Press, New York, pp. 167 - 176.

COULSON, D.B., FERGUSON, A.B. & DIEHL, R.C. (1966) Surg. Forum, 17 : 449 - 450. Effect of hyperbaric oxygen on the healing femur of the rat.

CRELIN, E.S. (1967) Anat. Rec. 157 : 354. An Autoradiographic Study of Endochondral Ossification in Vitro.

CRELIN, E.S., & KOCH, W.E. (1967) Anat. Rec. 158 : 473 - 483. An Autoradiographic study of Chondrocyte transformation into Chondroclasts and Osteocytes during Bone Formation in vitro.

de DUVE, C. (1959) "Lysosomes, a new group of cytoplasmic particles", in Subcellular Particles, (Ed. T. Hayashi), Ronald Press, New York, pp. 128 - 159.

DINGLE, J.T. (1961) Biochem. J. 79 : 509 - 512. Studies on the Mode of Action of Excess Vitamin A. Release of a Bound Protease by the Action of Vitamin A.

DINGLE, J.T., FELL, H.B., & COOMBS, R.R.A. (1967) Int. Arch. Allergy, 31 : 283 - 303. The breakdown of embryonic cartilage and bone cultivated in the presence of complement - sufficient antiserum. Biochemical changes and the role of the lysosomal system.

DZIEWIATKOWSKI, D.D. (1951) J. Exp. Med. 93 : 451 - 458. Radioautographic Visualization of Sulphur - 35 Disposition in the Articular Cartilage and Bone of Suckling Rats following Injection of labelled Sodium Sulphate.

ELLIS, H.A., & PEART, K.M. (1970) Brit. J. Exp. Path. 51 : 43 - 52. The Effects of Heparin and Dextran Sulphate on Cultured Mouse Limb Bones.

ENDO, H. (1960) Exp. Cell Res. 21 : 151 - 163. Ossification in Tissue Culture.

FELL, H.B., & DINGLE, J.T. (1963) Biochem. J. 87: 403 - 408. Studies on the mode of action of excess of Vitamin A. Lysosomal protease and the degradation of cartilage matrix.

FELL, H.B., & MELLANBY, E. (1952) J. Physiol. Lond. 116 : 320 - 349. The Effect of Hypervitaminosis A on Embryonic Limb-Bones cultivated in vitro.

FELL, H.B., & MELLANBY, E. (1955) J. Physiol. 127 : 427 - 446. The Biological Action of Thyroxine on Embryonic Bones Grown in Tissue Culture.

FELL, H.B., MELLANBY, E., & PELC (1956) J. Physiol. 134 : 179 - 188. Influence of Excess Vitamin A on the Sulphate Metabolism of Bone Rudiments grown in vitro.

FELL, H.B., & ROBISON, R. (1929) Biochem. J. 23 : 767 - 784. Growth, development, and phosphatase activity of embryonic avian femora and limb buds cultivated in vitro.

FELL, H.B., & THOMAS, L. (1961) J. Exp. Med. 114 : 343 -362 The Influence of Hydrocortisone on the action of excess Vitamin A on limb bone rudiments in culture.

FELL, H.B., & WEISS, L. (1965) Exp. Med. 121 - 551 - 560. The Effect of Antiserum, Alone and with Hydrocortisone, and Foetal Mouse Bones in Culture.

FELL, H.B., DINGLE, J.T. & COOMBS, R.R.A. (1966) "Recent experiments on the degradation and synthesis of bone and cartilage matrix in organ culture" in Fourth European Symposium on Calcified Tissues - International Congress Series No. 120, Excepta Medica Foundation, pp. 27 - 29.

FISHMAN, W.H. (1967) Methods in Biochem. Analysis. 15: 77 - 139. Determination of beta - glucuronidases.

FLANAGAN, B., & NICHOLS, G.J.R. (1968) Calcif. Tissue Res. Suppl. 51 - 51B . The Quantitative Measurement of Bone Resorption and Balance in vitro by analysis of Hydroxyproline Metabolism.

FOLLIS, R.H. (1951) Proc. Soc. Exp. Biol. & Med. 76 : 722 - 724. Effect of Cortisone on growing bones of rat.

FORSTHOEFEL, P.F. (1959) J. Morphol. 104 : 89 - 142. The Embryological Development of the Skeletal Effects of the Luxoid Gene in the Mouse, including its interactions with the Luxate Gene.

GAILLARD, P.J. (1955) Exp. Cell. Res. Suppl. 3 : 154 - 169. Parathyroid gland tissue and bone in vitro.

GAILLARD, P.J. (1961) "Parathyroid and Bone in Tissue Culture", in the Parathyroids, (ed. R.O. Greep & R.V. Talmage), pp. 20 - 45.

GINTER, E.K. (1966) Bull. Exp. Biol. Med. 61 : 303 - 305. Effect of the degree of differentiation on growth of the analagen of the long bones of the limbs in mouse embryo in vitro.

GOLDHABER, P. (1958) Arch. Pathol. 66 : 634 - 641. The Effect of Hyperoxia on bone resorption in tissue culture.

GOLDHABER, P. (1960) "Behaviour of Bone in Tissue Culture" in Calcification in Biological Systems, (ed. R.F. Sognnaes), Amer. Assoc. Advanc. Sci., Washington, D.C., pp. 349 - 372.

GOLDHABER, P. (1963) "Some Chemical Factors influencing Bone Resorption in Tissue Culture" in Mechanisms of Hard Tissue Destruction , (Ed. R.F. Sognnaes), Amer. Assoc. Advanc. Sci., Washington, D.C., pp. 609 - 636.

GOLDHABER, P. (1965) Science, 147 : 407 - 408. Heparin Enhancement of Factors stimulating Bone Resorption in Tissue Culture.

GOLDHABER, P. (1965) "Bone Resorption Factors, Cofactors, and Giant Vacuole Osteoclasts in Tissue Culture" in The Parathyroid Glands, (Ed. P.J. Gaillard, R.V. Talmage & A.M. Budy), Univ. of Chicago Press, 12 : pp. 153 - 169.

GORHAM, L.W., & WAYMOUTH, C. (1965) Proc. Soc. Exp. Biol. Med. 119 : 287 - 290. Differentiation in vitro of Embryonic Cartilage and Bone in a Chemically defined medium.

HALASZ, N.A., & STIER, H.A. (1966) "Effects of Hyperbaric Oxygenation on Tissues in Organ Culture" in Proceedings of the Third International Conference on Hyperbaric Medicine, (ed. K.W. Brown and B.G. Cox), publication 1404, Nat. Academy of Sciences, Washington, D.C., pp. 119 - 124.

HALL, B.K. (1970) Biol. Rev. 45 : 455 - 484. Cellular Differentiation in Skeletal Tissues.

HALME, J., & KIVIRIKKO, K.I., KAITILA, I., & SAXEN, L. (1969) Biochem. Pharmacol. 18 : 827 - 836. Effect of Tetracycline on Collagen Biosynthesis in Cultured Embryonic Bones.

HAMBLEN, D.L. (1968) J. Bone Jt. Surg. 50A : 1129 - 1141. Hyperbaric Oxygenation. Its Effect on Experimental Staphylococcal Osteomyelitis in Rats.

HANCOX, N.M. (1956) "The Osteoclast" in The Biochemistry and Physiology of Bone, (ed. G.A. Bourne), Academic Press, New York, pp. 212 - 250.

HANCOX, N.M., & BOOTHROYD, B. (1963) "Structure - function relationship in the Osteoclast" in Mechanisms of Hard Tissue Destruction, (ed. R.F. Sognnaes), Amer. Assoc. Advanc. Sci., Washington, D.C., pp. 497 - 514.

HARRISON, R.G. (1907) Proc. Soc. Exp. Biol. Med. 4 : 140 - 143. Observations on the living developing nerve fibre.

HEERSCHE, J.N.M., & VOOGD CAN DER STRAATEN, W.A.De., (1965) Proc. Kon. Ned. Akad. Wet. C68 :277 - 288. A Radioautographic and Radiometric Analysis of the influence of Parathyroid Extract on the Proline binding capacity of Cultivated Mouse Radius Rudiments.

HELLER - STEINBERG, M. (1951) Amer. J. Anat. 89 : 347 - 379. Ground Substance, Bone Salts and Cellular Activity in Bone Formation and Destruction.

HEPPLESTON, A.G., & SIMNETT, J.D. (1964) Lancet 1 : 1135 - 1137. The Tissue Reaction to Hyperbaric Oxygen.

HOLTROP, M.E. (1966) "The Origin of bone cells in endochondral ossification" in Proceedings Third European Symposium on Calcified Tissues, Davos, 1965, (ed. H. Fleisch, H.J.J. Blackwood, M. Owen), Springer-Verlag, Berlin, pp. 32 - 36.

HOOFF, A. van dan. (1964) Acta. Anat. (Basel) 57 : 16 - 28. Polysaccharide Histochemistry of Endochondral Ossification.

ITO, Y., & ENDO, H. (1956) Endocrinol. Japon. 3 : 106 - 115. Studies on the salivary gland hormones in tissue culture.

JACKSON, D.S. & CLEARY, E.G. (1967) Methods in Biochemical Analysis 15 : 26 - 56. The Determination of Collagen and Elasten.

JEFFREY, J.J. & MARTIN, G.R. (1966) Biochem. Biophys. Acta 12 : 269 - 280. The role of ascorbic acid in the biosynthesis of collagen.

JOHNSON, M.L. (1933) Amer. J. Anat. 52 : 241 - 271. The Time and Order of Appearance of Ossification Centre in the Albino Mouse.

JONES, H.H. & KEELER, D. (1971) Clin. Orth. 74 : 273 - 278. Organ Culture Method using a modified Grobstein Raft Technique.

KAUFMAN, E.J., GLIMCHER, M.J., MECHANIC, G.L., & GOLDHABER, P. (1965) Proc. Soc. Exptl. Biol. Med. 120 : 632 - 636. Collagenolytic Activity during Active Bone Resorption in Tissue Culture.

KODICEK, E. (1965) "The Effect of Ascorbic acid on Biosynthesis of components of connective tissue", in Structure and function of connective and skeletal tissue, (eds. S. Fitton Jackson, R.D. Harkness, S.M. Partridge and G.R. Tristram), pp. 307 - 318.

KUHLMAN, R.E. (1965) J. Bone Jt. Surg. 47A : 545 - 550. Phosphatases in Epiphyseal Cartilage : Their possible role in tissue synthesis.

LEWIS, E.A., & IRVING, J.T. (1970) Arch. Oral. Biol. 15 :769 - 776. An autoradiographic investigation of bone remodelling in the rat calvarium grown in organ culture.

LILLIE, R.D. (1965) "Staining References" in Histopathologic Technic and Practical Histochemistry, 3rd edition, The Blakiston Division, McGraw Hill Book Co., pp. 177, 506 - 507, 545.

LISKOVA, M., & JEAN, P. (1970) Exp. Cell. Res. 62 : 483 - 486. In vitro production of large osteoclasts by serum.

LUCY, J.A., DINGLE, J.T. & FELL, H.B. (1961) Biochem. J. 79 : 500 - 508. Studies on the Mode of Section of Excess of Vitamin A. A possible role of intracellular proteases in the degradation of cartilage matrix.

MANSPEIZER, S., & TONNA, E.A. (1967) Proc. Soc. Exp. Biol. Med. 124 : 599 - 603. The effects of extreme environmental oxygen tensions on the periosteal proliferation of mouse femora.

MAWHINNEY, B.S. (1968) Exp. Cell. Res. 51 : 301 - 312. Some physical and chemical factors affecting the in vitro growth of embryonic rat bone.

MAXIMOW, A. (1925) Contr. Embryol. Carneg. Instn. 16 : 49. Tissue Cultures of young mammalian embryos.

MITCHELL, J.R. (1950) Anat. Rec. 106 : 111 - 114. Growth of Embryonic Rat Bone in Circulating Medium.

MOORE, S., & STEIN, W.H. (1951) J. Biol. Chem. 192 : 663 - 681. Chromatography of amino acids on sulfonated polystyrene resins.

MOSCONA, M.H., & KARNOFSKY, D.A. (1960) Endocrinology 66 : 533 - 549. Cortisone induced modifications in the development of the chick embryo.

MOWRY, R.W. (1963) Ann. N.Y. Acad. Sci. 106 : 402 - 423. The Special Value of Methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins with revised directions for the colloidal iron stain, the use of alcian blue G8X and the combinations with the periodic acid-schiff reaction.

MUROTA, S., ENDO, H. & TAMAOKI, B. (1967) Biochem. Biophys. Acta. 136 : 379 - 385. Identification of Metabolites of Cortisol in Cultured Bone and their effects upon bone formation.

NAGAI, Y., LAPIERE, C.M. & GROSS, J. (1966) Biochemistry 5 : 3123 - 3130. Tadpole Collagenase, Preparation and Purification.

NEUMAN, R.E., & LOGAN, M.A. (1950) J. Biol. Chem. 184 : 299 - 305. The determination of hydroxyproline.

NIINIKOSKI, J., PENTTINEN, R., & KULONEN, E. (1970) Calc. Tiss. Res. 4 :(Suppl) 115 - 116. Effect of hyperbaric oxygenation on fracture healing in the rat : A biochemical study.

NIVEN, J.S.F. (1931) J. Path. Bact. 34 : 307 - 423. The Repair in vitro of Embryonic Skeletal Rudiments after Experimental Injury.

OWEN, M., (1956) J. Bone Jt. Surg. 38B : 762 - 769. Measurement of the Variations of Calcification in Normal Rabbit Bone.

OWEN, M. (1967) J. Cell. Sci. 2 : 39 - 56. Uptake of (^{3}H) uridine into precursor pools and RNA in Osteogenic cells.

PAFF, G.H. (1948) Proc. Soc. Exp. Biol. Med. 68 : 288 - 293. Influence of pH on growth of bone in tissue culture.

PAL, M.K., & SCHUBERT, M. (1962) J. Am. Chem. Soc. 84: 4384 - 4393. Measurement of the Stability of Metachromatic Compounds.

PECK, W.A., BRAND, T.J., & MILLER, I. (1967) Proc. Nat. Acad. Sci. 57: 1599 - 1606. Hydrocortisone - induced inhibition of protein synthesis and undine incorporation in isolated bone cells in vitro.

PERSSON, B.M. (1967) Acta. Orth. Scand. 38: 23 - 34. Effect of hyperbaric oxygenation on longitudinal growth of bones.

PERRINS, D.J.D., MAUDSLEY, R.H., COLWILL, M.R., SLACK, W.K., & THOMAS, D.A. (1966) 'OHP in the Management of Chronic Osteomyelitis" in Proceedings of the Third International Congress on Hyperbaric Medicine, (ed. I.W. Brown & B.G. Cox), Publication 1404 of the National Academy of Sciences, National Research Council, Washington, D.C.1966., pp. 578 - 584.

PROCKOP, D.J., & KIVIRIKKO, K.I. (1967) Annals. Int. Med. 66 : 1243 - 1266. Relationship of Hydroxyproline Excretion in Urine to Collagen Metabolism. Acceptance of use of Hydroxyproline as an indicator of collagen metabolism.

PROCKOP, D.J., & UDENFRIEND, S. (1960) Anal. Biochem. 1 : 228 - 239. A specific method for the analysis of Hydroxyproline in tissues and urine.

PROFITT, W.R., & ACKERMAN, J.L. (1964) Science 145: 932 - 934. Fluoride : Its Effects on Two Parameters of Bone Growth in Organ Culture.

QUINTARELLI, G. (1968) "Methods for the Histochemical Identification of Acid Mucopolysaccharides: A Critical Evaluation" in The Chemical Physiology of Mucopolysaccharides, (ed. Guiliano Quintarelli), Boston, Little, Brown & Co., pp. 199 - 215.

RACE, G.J., MATTHEWS, J.L., FINNEY, J.W., & MARTIN, J.H. (1969) "Ultrastructural Changes in Rat Adrenal Cortex following Hyperbaric Oxygen" in Proc. of Fourth Int. Cong. Hyperbaric Medicine, (ed. J. Wada & T. Iwo), Igaku Shoin Ltd., Tokyo. pp. 170 - 178.

RAJAN, K.T. (1969) Exp. Cell. Res. 55 : 419 - 422. The Cultivation in vitro of Post-Foetal Mammalian Cartilage and its Response to Hypervitaminosis A.

RAMALINGAM, K., & RAVINDRANATH, M.H. (1971) Stain Techn., 46 : 221 - 226. Effects of Ethanol on the Metachromatic Reaction of Tolvidine Blue O.

RAMP, W.K., & NEUMAN, W.F. (1971) Amer. J. Physiol. 220 : 270 - 274. Some factors affecting mineralization of bone in tissue culture.

RAMP, W.K., & THORNTON, P.A. (1968) Calc. Tiss. Res. 2 : 77 - 82. The Effect of Ascorbic Acid on the Glycolytic and Respiratory Metabolism of Embryonic Chick Tibia.

REYNOLDS, J.J. (1966) Exp. Cell. Res. 41 : 174 - 189. The Effect of Hydrocortisone on the growth of chick bone rudiments in chemically defined medium.

REYNOLDS, J.J. (1966) Exp. Cell. Res. 42 : 178 - 188. The Effect of Ascorbic Acid on the growth of chick bone rudiments in chemically defined medium.

REYNOLDS, J.J. (1967) Exp. Cell. Res. 47 : 42 - 48. The Synthesis of collagen by chick bone rudiments in vitro.

ROSENBERG, L. (1971) J. Bone Jt. Surg. 53A : 69 - 82. Chemical Basis for the Histological Use of Safranin O in the Study of Articular Cartilage.

SAXEN, L. (1966) J. Exp. Zool. 162 : 269 - 294. Effect of Tetracycline on Osteogenesis in Vitro.

SCHRYVER, H.F. (1965) Exp. Cell. Res. 40 : 610 - 618. The Effect of Hydrocortisone on Chondroitin sulphate production and loss by embryonic chick tibiotarsus in organ culture.

SCHRYVER, H.F. (1966) J. Exp. Zool. 162 : 81 - 88. A Quantitative Comparison of the Growth of the Embryonic Chick Tibiotarsus in vivo and in vitro.

SCHWARTZ, P.L., WETTENHALL, R.E.H., & BORNSTEIN, J. (1968) J. Exp. Zool. 168 : 517 - 530. The Growth of Newborn Rat Tibiae in a Continuous-flow Organ Culture System.

SELYE, H. (1958) Arthritis and Rheumatism 1 : 87 - 90. Sensitisation of the skeleton to vitamin A overdosage by cortisol.

SEVERSON, A.R. & TONNA, E.A. (1968) Proc. Soc. Exp. Biol. 129 : 315 - 319. The Effect of depressed environmental oxygen tensions on the periosteal proliferation of Mouse Femora.

SHAW, J.L., & BASSETT, C.A.L. (1967) J. Bone Jt. Surg. 49A : 73 - 80. The effects of varying oxygen concentrations on osteogenesis and embryonic cartilage in vitro.

SHIMIZU, M., GLIMCHER, M.J., TRAVIS, D., GOLDHABER, P. (1969) Proc. Soc. Exp. Biol. Med. 130 : 1175 - 1180. Mouse Bone Collagenase : Isolation, Partial Purification and Mechanism of Action.

SLACK, W.K., THOMAS, D.A., & PERRINS, D. (1965) Lancet, 1 : 1093 - 1094. Hyperbaric Oxygenation in Chronic Osteomyelitis.

SLEDGE, C.B. (1968) Clin. Orthop. 61 : 37 - 47. Biochemical Events in the Epiphyseal Plate and their Physiological Control.

SLEDGE, C.B., & DINGLE, J.T. (1965) Nature 205 : 140 - 143. Oxygen induced Resorption of Cartilage in Organ Culture.

SPICER, S.S. (1963) Ann. N.Y. Acad. Sci. 106 : 379 - 388. Histochemical differentiation of mammalian mucopolysaccharides.

STEGEMANN, H. (1958) Z. Physiol. Chem. 311 : 41 - 45. Mikrobestimmung von hydroxyprolin mit Chloramin -T und p - Dimethylamino - benzaldehyd.

STEIN, W.H., & MOORE, S. (1950) Cold Spring Harbor Symposia Quant. Biol. 14 : 179 - 190. Chromatographic determination of the amino acid composition of proteins.

STERN, B.D., MECHANIC, G.L., GLIMCHER, M.J., & GOLDHABER, P. (1963) Biochem. Biophys. Res. Comm. 13 : 137 - 143. The resorption of bone collagen in tissue culture.

STERN, B., GLIMCHER, M.J., & GOLDHABER, P. (1966) Proc. Soc. Exp. Biol. Med. 121 : 869 - 872. The effect of various oxygen tensions on the synthesis and degradation of bone collagen in tissue culture.

SUSI, F.R., GOLDHABER, P., & JENNINGS, J.M. (1966) Amer. J. Physiol. 211 : 959 - 962. Histochemical and Biochemical Study of Acid Phosphatase in Resorbing Bone in Culture.

TEAFORD, M.A., & WHITE, A.A. (1964) Proc. Soc. Exp. Biol. Med. 117 : 536 - 541. Culture Medium for Suitable Growth and Development of Embryonic Chick Femora in vitro.

TOLNAI, S. (1968) Can. J. Physiol. Pharmacol. 46 : 261 - 267. Effects of parathyroid hormone on bone acid hydrolases in tissue culture.

TONNA, E.A. (1961) J. Biophys. Biochem. Cytol. 9 : 813 - 824. The Cellular Complement of the Skeletal System studied Autoradiographically with Tritiated Thymidine during Growth and Aging.

TORRIANI, A. (1960) Biochem. Biophys. Acta. 38 : 460 - 479. Influence of inorganic phosphate in the formation of phosphatases by Escherichia coli.

TROWELL, O.A. (1958) Exp. Cell. Res. 16 : 118 - 147. The Culture of Mature Organs in a Synthetic Medium.

TRUETA, J. (1963) J. Bone Jt. Surg. 45B : 402 - 418. The Role of the Vessels in Osteogenesis.

VAES, G. (1968) J. Cell. Biol. 39 : 676 - 697. On the Mechanisms of Bone Resorption, the Action of Parathyroid Hormone on the Excretion and Synthesis of Lysosomal Enzymes and on the Extracellular Release of Acid by Bone Cells.

VAES, G. (1969) "Lysosomes and the cellular physiology of bone resorption" in Lysosomes in Biology and Pathology Vol. 2 , (ed. J.T. Dingle and H.B. Fell), North Holland Publishing Co., Amsterdam. London, pp. 217 - 253.

VAES, G. & JACQUES, P. (1965) Biochem. J. 97 : 380 - 388. Studies on Bone Enzymes. The Assay of Acid Hydrolases and other Enzymes in Bone Tissue.

VERITY, M.A., CAPER, R., & BROWN, W.J. (1968) Biochem. J. 109 : 149 - 154. Effect of Cations on Structure - Linked Sedimentability of Lysosomal Hydrolases.

WATTIAUK, R. & de DUVE, C. (1956) Biochem. J. 63 : 606 - 608. Tissue Fractionation Studies. Release of Bound Hydrolases by means of Triton X - 100.

WEISSMAN, G., & DINGLE, J. (1961) Exp. Cell. Res. 25 : 207 - 210. Release of Lysosomal Protease by Ultraviolet Irradiation and Inhibition by Hydrocortisone.

WOESSNER, J.F. (1967) Federation Proc. , Fed. Am. Soc. Exp. Biol. 26 : 607. Digestion of Protein-Polysaccharide Complex by Cathepsin D.

WOLFF, E., HAFFEN, K., KIENY, M., & WOLFF, E. (1953) J. Embryol. Exp. Morphol. 1 : 55 - 84. Essais de cultures in vitro d'organes embryonnaires en milieux synthetiques.

WOODS, J.F., & NICHOLS, G. (1965) J. Cell. Biol. 26 : 747 - 757. Collagenolytic Activity in Rat Bone Cells.

WRAY, J.B., & ROGERS, L.S. (1968) J. Surg. Res. 8 : 373 - 378. Effect of hyperbaric oxygenation upon fracture healing in the rat.

YABLON, I.G., & CRUESS, R.L. (1968) J. Trauma 8 : 186 - 202. The Effect of Hyperbaric Oxygen on fracture healing in rats.

YOUNG, M.H., & CRANE, W.A.J. (1944) Ann. Rheum. Dis. 23 : 163 - 168. Effect of Hydrocortisone on the utilization of tritiated thymidine for skeletal growth in the rat.

ZANELLI, J.M., LEA, D.J. & NISBET, J.A. (1969) J. Endocr. 43 : 33 - 46. A Bioassay Method in Vitro for Parathyroid Hormone.

APPENDIX A

STAINING SCHEDULES

Mayer's Haematoxylin and Eosin (Lillie, 1965)

1. Paraffin sections are dewaxed in Xylene and brought through graded alcohols to water.
2. Stain in Mayer's Haematoxylin 5 - 10 minutes.
3. Rinse in distilled water.
4. Blue the sections in saturated lithium. carbonate solution 20 - 30 seconds.
5. Rinse in distilled water.
6. Stain in Eosin 30 seconds to 2 minutes.
7. Dehydrate through graded alcohols.
8. Clear in Xylene and mount.

Nuclei stain blue, cytoplasm, connective tissue and muscle stain pink.

Toluidine Blue - (Lillie, 1965)

1. Paraffin sections dewaxed in Xylene and taken down through graded alcohols to water.
2. Stain in Toluidine blue 1:1000 aqueous solution for 15 - 30 seconds.
3. Rinse in distilled water.
4. Blot dry with filter paper.
5. Clear in Xylene and mount.

Nuclei stain deep blue, cytoplasm light blue, cartilage matrix reddish-purple to violet.

Alcian blue - P.A.S. (Mowry, 1963)

1. Take paraffin sections through Xylene and graded alcohols to water.
2. Stain in 1% Alcian blue in 0.1N hydrochloric acid at pH 1.0 for 20 minutes.
3. Rinse for three minutes in running tap water and briefly in distilled water.
4. Oxidise in 1% aqueous Periodic acid for 10 minutes.
5. Wash in running tap water for 5 minutes and rinse in distilled water.
6. Treat with Schiff's reagent for 10 to 30 minutes.
7. Wash for at least 10 minutes in running tap water and dip briefly in distilled water.
8. Counterstain with Harris haematoxylin for 5 to 10 minutes.
9. Rinse briefly in running tap water to remove excess haematoxylin.
10. Dehydrate through graded alcohols, clear in Xylene and mount.

Acid mucopolysaccharides stain turquoise blue, neutral polysaccharides magenta and nuclei reddish-brown.

Heidenhain's Azan stain (Lillie, 1965)

1. Paraffin sections dewaxed in Xylene and passed down through graded alcohols to water.
2. Stain in Azocarmine B at 56°C to 60°C for 10 minutes.
3. Wash in distilled water.
4. Differentiate in .5% analine for 15 minutes.
5. Rinse in 1% acetic acid to stop differentiation.
6. Mordant in 5% phosphotungstic acid for 45 minutes.
7. Rinse in distilled water.
8. Stain in Analine blue-Orange G for 40 minutes.
9. Wash quickly in distilled water.
10. Dehydrate through graded alcohols, clear in Xylene and mount.

Young collagen, reticulin and basophil granules stain dark blue, cell nuclei and red cells red, and muscle and acidophil granules orange-red.

Methylene Blue - azure A - basic fuchsin (Belanger, 1961)

For poststaining after processing of autoradiographs.

Stain made up 0.065 g. methylene blue, add 0.010g. azure A, 5 mls. glycerine, 5 mls. methanol, 25 mls. distilled water and 15 mls. phosphate buffer solution (pH 6.9).

Maintain all solution at 4° C.

1. Stain processed autoradiograph slides in above stain for 5 mins.
2. Rinse in distilled water to remove excess stain.
3. Destain the emulsion in 0.5% aqueous sodium bisulphite, 5 - 8 mins.
4. Wash the slides for 2 mins. in distilled water.
5. Counter-stain in 0.05% aqueous basic fuchsin for 10 - 15 mins.
6. Remove excess stain in absolute alcohol (for 30 secs.).
7. Dehydrate in cold absolute alcohol for 5 mins. and in two other alcohol baths at room temperature, 5 mins. in each.
8. Clear and mount.

APPENDIX B

Composition of Synthetic Tissue Culture Media

1. Eagle's Basal Medium

	mg/100 ml.
Sodium chloride (NaCl)	680.0
Potassium chloride (KCl)	40.0
Calcium chloride ($CaCl_2$)	20.0
Magnesium sulphate ($MgSO_4.7H_2O$)	20.0
Sodium phosphate ($NaH_2PO_4.2H_2O$)	15.0
l-arginine hydrochloride	2.1
l-cystine	1.2
l-histidine hydrochloride	0.95
dl-isoleucine	5.2
dl-leucine	5.2
l-lysine hydrochloride	3.6
dl-methionine	1.5
dl-phenylalanine	3.3
dl-threonine	4.8
dl-tryptophane	0.8
l-tyrosine	1.8
dl-valine	4.7
Aneurine hydrochloride	0.1
Choline chloride	0.1
Folic acid	0.1
Inositol	0.2
Nicotinamide	0.1
Calcium pantothenate	0.1
Pyridoxal hydrochloride	0.1
d-biotin	0.1
Riboflavin	0.01
Glucose	100.0
l-glutamine	29.2
Phenol red	1.0

2. Medium 199

	mg/100 ml.
Sodium chloride (NaCl	680.0
Potassium chloride (KCl)	40.0
Calcium chloride ($CaCl_2$)	20.0
Magnesium sulphate ($MgSO_4.7H_2O$)	20.0
Sodium phosphate ($NaH_2PO_4.2H_2O$)	15.0
Ferric nitrate ($Fe(NO_3)_3.9H_2O$)	0.07
l-arginine hydrochloride	7.0
l-histidine hydrochloride	2.0
l-lysine hydrochloride	7.0
dl-phenylalanine	5.0
dl-methionine	3.0
dl-serine	5.0
dl-threonine	6.0
dl-leucine	12.0
dl-isoleucine	4.0
dl-valine	5.0
dl-glutamic acid	15.0
dl-aspartic acid	6.0
dl-alanine	5.0
l-proline	4.0
l-hydroxyproline	1.0
glycine	5.0
dl-tryptophane	2.0
l-tyrosine	4.0
l-cystine	2.0
l-cysteine hydrochloride	0.01
Aneurine hydrochloride	0.001
Riboflavin	0.001
Pyridoxine hydrochloride	0.0025
Pyridoxal hydrochloride	0.0025
Nicotinic acid	0.0025
Nicotinamide	0.0025
Calcium pantothenate	0.001
Inositol	0.005
p-amino-benzoic acid	0.005
Choline chloride	0.05
Ascorbic acid	0.005
d-biotin	0.001
Folic acid	0.001
Menaphthone	0.001
Calciferol	0.01
Vitamin A acetate	0.01
a-Tocopheral phosphate	0.001

Medium 199 (continued)

	mg/100 ml.
Cholesterol	0.02
Adenine hydrochloride	0.51
Guanine hydrochloride	0.03
Xanthine	0.03
Hypoxanthine	0.03
Thymine	0.03
Uracil	0.03
Adenylic acid	0.02
Adenosine triphosphate	0.5
Ribose	0.05
Deoxyribose	0.05
Sodium acetate ($3H_2O$)	8.4
Glucose	100.0
Phenol red	1.0
Glutathione	0.005
l-glutamine	10.0
Tween 80	0.05

3. BGJ Medium (P6 Modification)

	mg/100 ml.
l-lysine. HCl	24.0
l-histidine. HCl.H_2O	15.0
l-arginine. HCl	7.5
l-threonine	7.5
l-valine	6.5
l-leucine	5.0
l-isoleucine	3.0
l-methionine	5.0
l-phenylalanine	5.0
l-tryptophan	4.0
l-tyrosine	4.0
l-cysteine. HCl.H_2O	9.0
l-glutamine	20.0
Potassium chloride	35.0
Potassium dihydrogen phosphate	3.5
Disodium hydrogen phosphate	14.0
Sodium chloride	620.0
Sodium bicarbonate	220.0
Nicotinic acid	2.0
Thiamine. HCl	0.2
Calcium pantothenate	0.05
Riboflavin	0.02
Pyridoxal phosphate	0.02
Folic acid	0.02
Biotin	0.02
p-Aminobenzoic acid	0.2
- Tocopherol phosphate. Na	0.1
Choline chloride	5.0
m-Inositol	0.1
Vitamin B_{12}	0.004
Sodium acetate. $3H_2O$	8.3
Calcium lactate. $5H_2O$	55.5
Magnesium sulphate. $7H_2O$	20.0
Glucose	500.0
Streptomycin	5.0
Penicillin G	5000 units
Ascorbic acid	15.0

APPENDIX C

STATISTICAL ANALYSIS OF RESULTS

The results obtained for the elongation of limb bone rudiments in culture under varying experimental conditions were subjected to further statistical analysis to determine their level of significance. Because of the small numbers of paired rudiments involved the Student's "t" test was used. This calculates the ratio of the observed difference between the means of each set of increases in length, to the calculated standard error of that difference.

1) Effect of Gas Phase on Percentage Increase in Length of Tibial Rudiments

a) Comparison of 95% Air and 95% Oxygen (see Table 7, page 91).

(i) After 2 days

Rudiments in air - mean 9.2; standard deviation 4.7; standard error 1.4.

Rudiments in oxygen - mean 5.9; standard deviation 5.0; standard error 1.4.

t value 1.64 ; $p > 0.10$

The observed difference is not significant.

(ii) After 4 days

Rudiments in air - mean 14.1; S.D. 5.6; S.E. 1.6

Rudiments in oxygen - mean 5.6; S.D. 3.6; S.E. 1.0

t value 4.38 ; $p < 0.002$

The observed difference is highly significant.

(iii) After 6 days

Rudiments in air - mean 15.5; S.D. 5.4; S.E. 1.6

Rudiments in oxygen - mean 6.0; S.D. 5.2; S.E. 1.5

t value 4.37 ; $p < 0.002$

The observed difference is highly significant.

b) Comparison of 95% Air and 98% Oxygen at Hyperbaric Pressure (HBO).

(i) After 2 days

Rudiments in air - mean 9.2; S.D. 4.7; S.E. 1.4

Rudiments in HBO - mean 5.4; S.D. 3.7; S.E. 1.1

t value 2.19 ; $p < 0.05$

The observed difference is just significant.

(ii) After 4 days

Rudiments in Air - mean 14.1; S.D. 5.6; S.E. 1.6

Rudiments in HBO - mean 5.4; S.D. 3.2; S.E. 0.9

t value 4.65 ; $p < 0.001$

The observed difference is highly significant.

(iii) After 6 days

Rudiments in air - mean 15.5; S.D. 5.4; S.E. 1.6

Rudiments in HBO - mean 4.6; S.D. 2.6; S.E. 0.8

t value 6.29 ; $p < 0.001$

The observed difference is highly significant.

c) Comparison of 95% Oxygen and Hyperbaric Oxygen (HBO).

(i) After 2 days

Rudiments in oxygen - mean 5.9; S.D. 5.0; S.E. 1.4

Rudiments in HBO - mean 5.4; S.D. 3.7; S.E. 1.1

t value 0.31 ; $p > 0.1$

The observed difference is not significant.

(ii) After 4 days

Rudiments in oxygen - mean 5.6; S.D. 3.6; S.E. 1.0

Rudiments in HBO - mean 5.4; S.D. 3.2; S.E. 0.9

t value 0.19 ; $p > 0.1$

The observed difference is not significant.

(iii) After 6 days

Rudiments in oxygen - mean 6.0; S.D. 5.2; S.E. 1.5

Rudiments in HBO - mean 4.6; S.D. 2.6; S.E. 0.8

t value 0.82 ; $p > 0.1$

The observed difference is not significant.

2) Effect of Hydrocortisone 1.0 µg/ml on percentage increase in length of "Early" Tibial Rudiments (Table 16, page 159).

a) In 95% Air

(i) At 2 days

In plain medium - mean 9.3; S.D. 2.9; S.E. 1.7

With Hydrocortisone - mean 16.8; S.D. 2.4; S.E. 1.4

t value 3.43 ; $p < 0.05$

The observed difference is just significant.

(ii) At 4 days

In plain medium - mean 11.7; S.D. 2.0; S.E. 1.2

With Hydrocortisone - mean 16.5; S.D. 3.2; S.E. 1.8

t value 2.20 ; $p > 0.1$

The observed difference is not significant.

(iii) At 6 days

In plain medium - mean 16.3; S.D. 8.7; S.E. 5.0

With Hydrocortisone - mean 15.8; S.D. 7.1; S.E. 4.1

t value 0.08 ; $p > 0.1$

The observed difference is not significant.

b) In 95% Oxygen

(i) At 2 days

In plain medium - mean 7.9; S.D. 5.6; S.E. 3.2

With Hydrocortisone - mean 11.5; S.D. 6.0; S.E. 3.5

t value 0.76 ; $p > 0.1$

The observed difference is not significant.

(ii) At 4 days

In plain medium - mean 11.7; S.D. 6.9; S.E. 4.0

With Hydrocortisone - mean 15.5; S.D. 2.7; S.E. 1.6

t value 0.88 ; $p > 0.1$

The observed difference is not significant.

(iii) At 6 days

In plain medium - mean 11.7; S.D. 7.0; S.E. 4.0

With Hydrocortisone - mean 12.9; S.D. 1.7; S.E. 1.0

t value 0.30 ; $p > 0.1$

The observed difference is not significant.

c) In 98% hyperbaric oxygen (HBO)

(i) At 2 days

In plain medium - mean 4.6; S.D. 1.7; S.E. 1.0

With Hydrocortisone - mean 13.2; S.D. 1.2; S.E. 0.7

t value 6.87 ; $p < 0.02$

The observed difference is just significant.

(ii) At 4 days

In plain medium - mean 5.2; S.D. 3.3; S.E. 1.9

With Hydrocortisone - mean 6.2; S.D. 2.2; S.E. 1.3

t value 0.42 ; $p > 0.1$

The observed difference is not significant.

(iii) At 6 days

In plain medium - mean 6.7; S.D. 2.5; S.E. 1.5

With Hydrocortisone - mean 7.2; S.D. 3.1; S.E. 1.8

t value 0.19 ; $p > 0.1$

The observed difference is not significant.